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TITLE:  A New Therapeutic Paradigm for Breast Cancer Exploiting Low Dose Estrogen-Induce Apoptosis


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**14. ABSTRACT** To discover the mechanism of estrogen induced breast cancer cell apoptosis and establish the clinical value of short-term low dose estrogen treatment to cause apoptosis in antihormone resistance breast cancer. To achieve the goal, we have created an optimal collaborative network to study laboratory models of the regulation of estrogen-induced growth and apoptosis in breast cancer. The molecular mechanisms of estrogen action (ER) mediated regulation are being deciphered by the systematic distribution of processed tissues from the Fox Chase Cancer Center (FCCC) to Translational Genomics (TGen) for genomics (siRNA analysis, CGH, and Agilent gene array) and to Georgetown University (GU) for proteomic analysis. All derived data is being loaded on a secure website for analysis. A complimentary clinical trial is currently recruiting to evaluate the antitumor effects of high dose estradiol (30 mg daily) in patients following the success and failure of two consecutive antihormonal therapies. We have published our findings about a new secreted protein, CEACAM-6 in estrogen-deprived breast cancer cells that enhances tumor invasion. We report our studies of the regulation of the antiapoptotic protein Bcl-2 by the estrogen induced protein XBP-1. This protein is overexpressed in our antihormone resistant cell lines as is Bcl-2.

**15. SUBJECT TERMS**
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**BC050277 – "A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis".**

## Introduction

The Center of Excellence Grant will complete four independent, interconnected and synergistic tasks to achieve the goal and answer the overarching question: **to discover the mechanism of estrogen induced breast cancer cell apoptosis and establish the clinical value of short-term low dose estrogen treatment to cause apoptosis in antihormone resistant breast cancer.** To achieve the goal, we have established an integrated organization **(Figure 1)** with a first class advisory board that links clinical trials **(Task 1)** with laboratory models and mechanisms **(Task 2)** proteomics **(Task 3)** and genomics **(Task 4).**
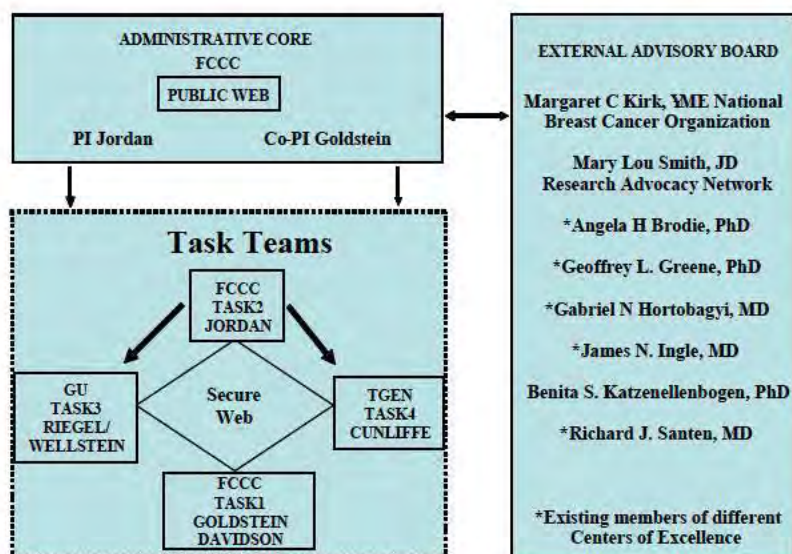


**Figure 1. Organization of the COE.**

## Body

The body will report our exceptional progress starting with the Administrative Core and then reports from the four Task teams.

## Administration and Data Tracking

The COE is generating large quantities of data of diverse types that need to be communicated to the participating COE investigators **(Figure 2)**. As a result, we created the Biostatistics and Bioinformatics Consortium Core (BBCC) to provide rigorous experimental design, data analysis and bioinformatics support to all COE investigators and physicians, and provide a means for information communication. The BBCC also provides tools for data access, management, annotation and publishing. The combination of this experienced group of biostatisticians, bioinformaticians and data management experts from FCCC (Dr. Ross, Dr. Litwin, Dr. Peri, Mr. Slifker, Ms. Tchuvatkina, Mr. Bland, Mr. Collins), Georgetown (Drs. Wu, Seillier-Moiseiwitsch, Ressom, Hu, Huang) and TGen (Drs. Bittner, Kim, Suh, Balagurunathan) will provide a cohesive core that effectively serves COE scientists and physicians. These individuals are knowledgeable about cancer biology, genetics, and epidemiology; and have broad experience in quantitative applications for clinical trials, pre-clinical studies, functional genomics, proteomics, translational investigations and cancer prevention and control research.
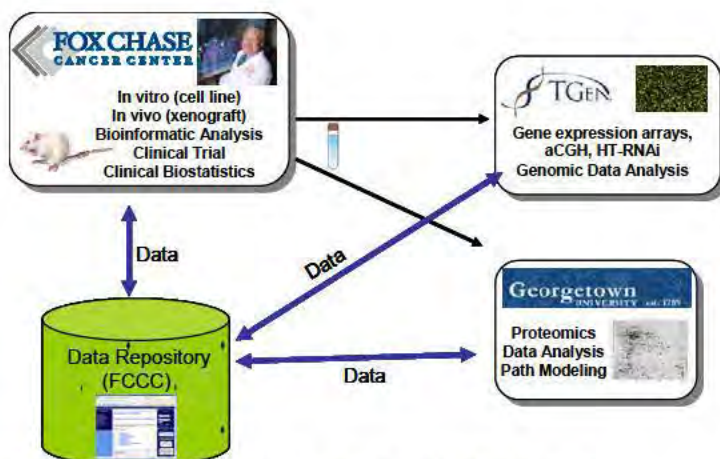
**Figure 2. Information flow in the COE project.**

## Accomplishments

Over the past 12 month BBCC continued to support the COE project research

- The BBCC maintained the COE web-portal (coe.fccc.edu) to enable the four geographically separate institutions to function in a more unified way by allowing timely sharing of research data and enhancing day-to-day communications among COE investigators (see figure). The portal has public and private components. The public component includes a description of each project/core and links to the participating organizations. Access to the private component of the portal is controlled through a robust, role-based security system. Restrictions are applied to each user commensurate with their needs to access the data. As studies expand, these various privileges will be reviewed and modified as needed. The secure portion of the portal provides a number of critical capabilities to the COE. These include:
    - Data repository to facilitate information collection/sharing and investigator collaboration. The SFTP component was added to the COE Data Repository (COE-DR) to provide secure storage of large quantities of high-throughput genomic and proteomic data. Each institution can use COE-DR to store data with or without sharing them with other collaborators. FCCC has access to both private and shared files provided by all 3 institutions.
    - Work Group Collaboration and Communication Tools: These portal features facilitate communications and cooperative work among geographically diverse COE participants. Among the materials added to the site are presentations from the last year's review which are available to advisers and the COE collaborators.
- The BBCC collaborated with COE investigators in the design of SOPs for clinical sample identification. In the COE project each sample has a unique identifier composed of the study name (COE/E2-RARE) and a unique sample number. Both study and sample IDs are displayed in human readable form and as 1D-bar codes on a sample label **(Figure 3)**. The first batch of clinical sample labels was printed in preparation for the upcoming clinical trial. Samples collected through clinical investigations will present sample type (serum, plasma, frozen biopsy, biopsy formalin block), site number, patient sequence number, study timepoint and date and time of collection.
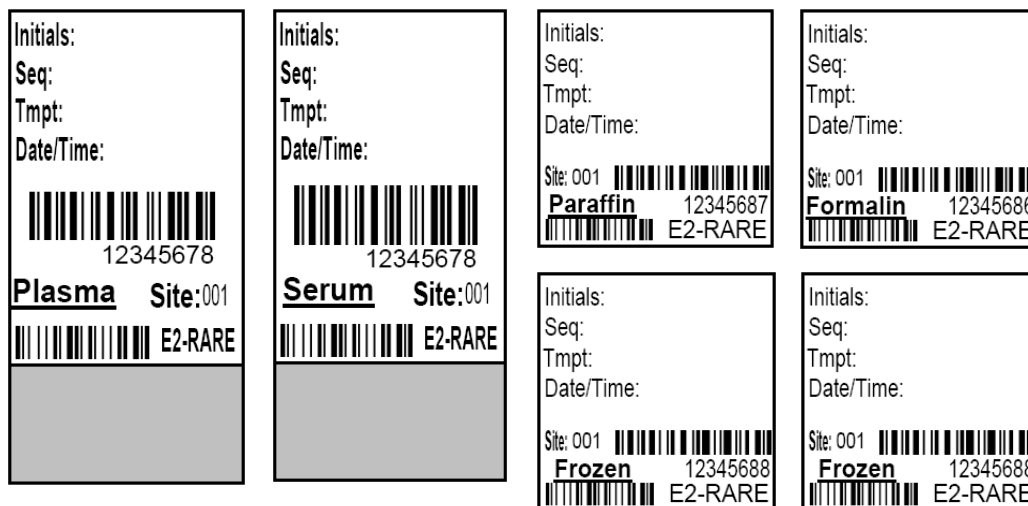
**Figure 3. Examples of clinical sample labels for data tracking.**

- The BBCC designed and developed the first version of the information system for the clinical trial of estradiol in postmenopausal women with hormone receptor-positive metastatic breast cancer exhaustively treated with antihormone therapy. This system accommodates the collection and storage of information generated by the Clinical Trials Consortium studies including: enrollment logs, patient demographics, health history, physical exams, prior treatment, concomitant medications, drug compliance, adverse events/toxicities, clinical responses, clinical labs, quality of life, and blood/biopsy samples. Clinical sample registration functionality was implemented using the identification scheme described above. The web interface was designed to be used in conjunction with a laser bar-code label scanner. This approach will improve data entry efficiency, and quality control by minimizing transcription errors. In addition extensive data validations have been incorporated to the user-interfaces to improve data entry accuracy. This web-based application is built using J2EE technologies.
- The BBCC provided support and maintenance for the COE experimental sample information system. This J2EE multi-tier application accommodates the collection and storage of information generated by the cell culture experiments conducted at FCCC. This information includes sample availability, sample location, quality control measurements and information about sample shipping **(Figure 4)**. A more complete description of experiments conducted using this system can be found in the Task 2a (FCCC/Jordan, Ariazi) section of this report.
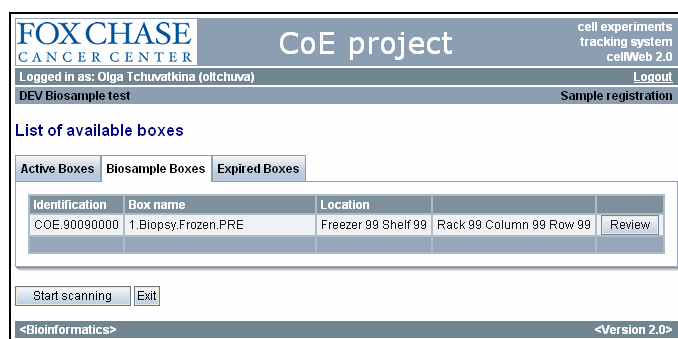


**Figure 4. Screenshot of the COE experimental sample tracking system.**

- The BBCC started design and development of an information system to support COE clinical samples experiments. This year a first version of a clinical samples registration module was added to the culture experiments information system. This module will provide functionality for migrating clinical samples from the Clinical Trials Consortium storage to the research laboratory for further analysis. The resulting

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system will provide the same functionality for clinical samples as is already available for cell culture experiments.

- The BBCC conducted periodic web-conferences. The purpose of these web-meetings was to build collaborations, exchange data analysis and data management capabilities, ensure coordination of biostatistical and bioinformatics efforts across the several COE institutions, and define requirements for the COE-DR. Each conference lasted 1 hour and included a formal presentation by one of the sites followed by group discussion. A commercial web-meeting software product (www.livemeeting.com) was used to augment voice conference calls via simultaneous video of the presentation (e.g., software demonstration, PowerPoint slides) on their desktop computers via internet connections. Agendas and supporting materials are available on the secure portion of the COE portal.

- The BBCC performed extensive analysis of tumor xenograft microarray data. Open-source analytical tools (R/Bioconductor) were used for QC analysis, annotation, visualization, differential expression analysis and enrichment analysis of microarray data representing a variety of experimental conditions. Separate analyses were performed on data from Agilent and Affymetrix platforms, and cross-validated gene lists were used for downstream analysis whenever possible. Details of experimental design and analysis of xenograft tumor microarray data appears under Task 4a (FCCC/Jordan, Ariazi).

- The BBCC developed an automated graphing tool to produce on-the-fly time-course gene expression profiles. Expression measures from a total of 82 microarrays representing gene expression for two cell lines (MCF-7/WS8 and MCF-7/5C) across 7 time points with 6 replicates per time point were summarized into a single Excel workbook. A Visual Basic macro was written which allows the on-the-fly creation of expression profile plots for any selected gene (**Figure 5**). This has proved useful for quickly assessing expression profiles of candidate genes of interest.
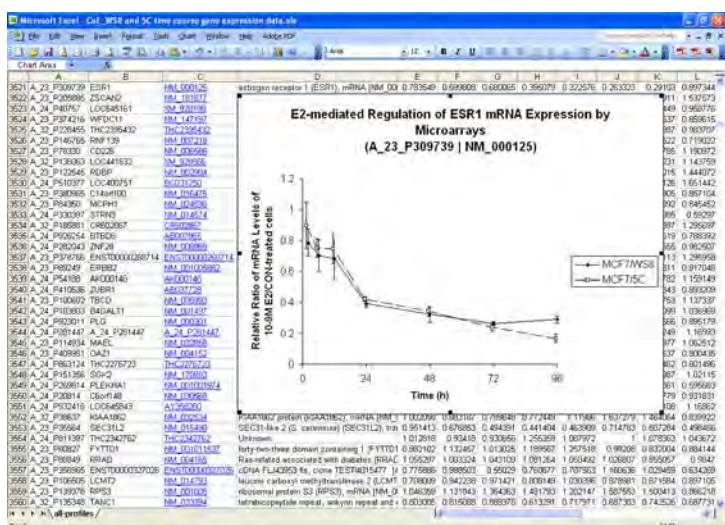


**Figure 5. Screenshot of the cell line microarray database and automated graphing tool.**

- The BBCC acquired and supports GeneGo Metacore Analysis Suite of bioinformatic systems biology applications. GeneGo Metacore applications facilitates analyses of gene expression microarray data and other types of 'omics' systems biology data by mining for cell signaling and regulatory pathways in the experimental data. GeneGo maintains a manually curated database of experimentally validated protein-protein, protein-DNA and protein-compound interactions and integrates this information to build canonical signaling and metabolic pathways. The Metacore applications implement this biological database with advanced algorithms to analyze and visualize broad types of systems level experimental data. We have applied GeneGo Metacore applications to analyze the *in vivo* antihormone-resistant breast cancer tumor models as described under Task 4.

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**Task 1.   To conduct exploratory clinical trials to determine the efficacy and dose response of pro-apoptotic effects of estrogen [Estrace] in patients following the failure of two successful antihormonal therapies.**

Task 1a (FCCC, Goldstein/Swaby): To confirm the efficacy of standard high dose estrogen (Estrace) therapy and then determine a minimal dose to induce tumor regression.

**TASK 1a - FCCC/Goldstein, Swaby – Clinical trial conducted by Dr. Ramona Swaby under direction of Dr. Lori Goldstain at FCCC**

Here we report work completed on Tasks 1a at the Fox Chase Cancer Center site during year 2 of this COE involving the Reversal of Antihormone Resistance by Estrogen clinical trial .

**DOSE DE-ESCALATION OF ESTROGEN (ESTRACE) TO REVERSE ANTIHORMONE RESISTANCE IN PATIENTS ALREADY EXHAUSTIVELY TREATED WITH ANTIHORMONE THERAPY**

**Task 1a.**
During the second year of funding, we have built the clinical infrastructure for the conduct of this multi-institutional clinical trial associated with the award. We have successfully secured funding for this investigator-initiated clinical trial as a non-restricted grant from Astra-Zeneca Pharmaceuticals to financially support the clinical trial operations. These funds will support the Fox Chase Cancer Center protocol support management office which will serve as the functional "central operations center" for the adverse event monitoring and regulatory surveillance and control, as well as quality assurance of the clinical trial. As such, the protocol support management team has created a portfolio of case report forms enabling reporting of adverse events, patient enrollment logs, pill diary forms, as well as recording measurement of response to treatment. Additionally, in collaboration with the Fox Chase Cancer Center Biostatistics department, we have developed an electronic database for the clinical information acquisition including patient enrollment logs and demographics, health history, physical exams, prior treatment(s), concomitant medications, drug compliance, adverse events/toxicities, clinical responses, clinical labs and quality of life assessments.

The clinical trial has now been approved by the Fox Chase Cancer Center Institutional Review Board and approved by the grant committee of Astra-Zeneca Pharmaceuticals (March 2007) in accordance with providing funding. The Department of Defense approved the protocol (March 27, 2008), and we have now started to screen and accrue patients. In the past few months, we have screened 8 patients. The first patient is enrolled and currently being treated and is tolerating therapy well without significant toxicities. Therefore, we are in the process of expanding our eligibility criteria and our network of collaborating hospitals to facilitate recruitment and successful accrual.

In conclusion, we believe that these accomplishments during the second years of this COE have provided the financial, regulatory and electronic infrastructure to successfully conduct the clinical trial examining a new therapeutic paradigm for breast cancer exploiting low dose estrogen to induce apoptosis and reverse resistance to anti-estrogen therapy.

**TASK 2. To elucidate the molecular mechanism of $E_2$ induced survival and apoptosis in breast cancer cells resistant to either selective ER modulators (SERMs) or long-term estrogen deprivation.**

Task 2a (FCCC, Jordan/Ariazi): To complete a series of experiments using sets of well defined breast cancer models of $E_2$-induced survival and apoptosis *in vivo* and *in vitro* [at the Fox Chase Cancer Center (FCCC)]. FCCC will generate protein samples for proteomic analyses [carried out] under Task 3 [at Georgetown University (GU)] and RNA samples for gene expression microarray analyses [carried out] under Task 4 [at Translational Genomics Research Institute (TGen)].

Task 2b (FCCC, Jordan/Lewis-Wambi and Sengupta): To confirm and validate developing pathways of $E_2$-induced breast cancer cell survival and apoptosis.

**TASK 2a - FCCC/Jordan, Ariazi - Studies carried out by Dr. Eric Ariazi in the Jordan laboratory at FCCC**

**Task 2a (FCCC/ Jordan, Ariazi): To complete a series of experiments using sets of well defined breast cancer models of $E_2$-induced survival and apoptosis *in vivo* and *in vitro* [at the Fox Chase Cancer Center (FCCC)]. FCCC will generate protein samples for proteomic analyses [carried out] under Task 3 [at Georgetown University (GU)] and RNA samples for gene expression microarray analyses [carried out] under Task 4 [at Translational Genomics Research Institute (TGen)].**

Here we report work completed on Tasks 2a at the Fox Chase Cancer Center site during year 2 of this COE involving generation of RNA and protein samples of the *in vitro* antihormone-resistant breast cancer cell line models.

**GENERATION OF CELL LINE SAMPLES FOR PROTEOMIC (UNDER TASK 3) AND MICROARRAY ANALYSES (UNDER TASK 4)**

**WORK ACCOMPLISHED**

**Experiments Completed During Year 1**

**Production of Proteomic Samples for Task 3**
*Experiment 1)* Production of MCF-7/WS8 protein samples for proteomics of cells treated plus/minus $10^{-9}$ M $E_2$ for a long-term time course in which cells were harvested at 24 h, 48 h, and 72 h.

*Experiment 2)* Production of MCF-7/5C protein samples for proteomics of cells treated plus/minus $10^{-9}$ M $E_2$ for a long-term time course in which cells were harvested at 24 h, 48 h, and 72 h.

**Production of Microarray Samples for Task 4**
*Experiment 3)* Production of MCF-7/WS8 RNA samples for microarrays of cells treated plus/minus $10^{-9}$ M $E_2$ for 2 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h.

*Experiment 4)* Production of MCF-7/5C RNA samples for microarrays treated plus/minus $10^{-9}$ M $E_2$ for 2 h, 6 h, 12 h, 24 h, 48 h and 96 h.

These previously completed experiments have been described in the Year 1 Progress Report for this award under Task 2a.

## Experiments Completed During Year 2

### Production of Proteomic Samples for Task 3

*Experiment 1)* Production of MCF-7/WS8 protein samples for proteomics of cells treated plus/minus $10^{-9}$ M $E_2$ for 2 h.

*Experiment 2)* Production of MCF-7/5C protein samples for proteomics of cells treated plus/minus $10^{-9}$ M $E_2$ for 2 h.

*Experiment 2)* Production of MCF-7/2A protein samples for proteomics of cells treated plus/minus $10^{-9}$ M $E_2$ for 2 h.

### Production of Microarray Samples for Task 4

*Experiment 4)* (Short-term time course) Production of MCF-7/2A RNA samples for microarrays of cells treated plus/minus $10^{-9}$ M $E_2$ for a relatively short-term time course in which cells were harvested at 2 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h.

*Experiment 5)* (Long-term time course) Production of MCF-7/2A RNA samples for microarrays treated plus/minus $10^{-9}$ M $E_2$ for a relatively long-term time course in which cells were harvested at 3 days, 4 days, 5 days, 6 day, 7 days, 8 days, and 9 days.

## Methods and Results

### Cell Lines

The cell lines used to generate microarray and proteomics samples were wild-type estrogen-responsive MCF-7/WS8 cells (1, 2), aromatase-inhibitor resistant MCF-7/5C cells (1, 3) which undergo $E_2$-induced apoptosis with fast kinetics (starts within 3 days), and aromatase-inhibitor resistant MCF-7/2A cells, which undergo $E_2$-induced apoptosis with slow kinetics (starts within 6 days) [see elsewhere under Task 2 (Lewis-Wambi); (2, 4). MCF-7/WS8 cells were maintained in fully estrogenized media (phenol red-containing RPMI-1640 and 10% whole fetal bovine serum (FBS), supplemented with 6 ng/ml insulin, 2 mM glutamine, 100 μM non-essential amino acids, and 100 U of penicillin and streptomycin per ml). MCF-7/5C and MCF-7/2A cells were maintained in estrogen-free media (phenol red-free RPMI-1640 and 10% dextran-coated charcoal-treated FBS (DCC-FBS) plus the same supplements as for fully estrogenized media). Cells were maintained at 37° C in a humidified 5% $CO_2$ atmosphere. Three days prior to an experiment, MCF-7/WS8 cells were switched to estrogen-free media.

### Experiments 1, 2, and 3: Production of protein samples for proteomic analysis under Task 3

Each of the cell lines, wild-type estrogen-responsive MCF-7/WS8, aromatase inhibitor-resistant MCF-7/5C and aromatase inhibitor-resistant MCF-7/2A cells, was seeded into twenty 15-cm plates at 70% confluency using estrogen-free RPMI-1640 media supplemented with 10% DCC-FBS. The estrogen-responsive MCF-7/WS8 cells had been cultured under estrogen-free conditions for 3 days prior to seeding, while the MCF-7/5C and MCF-7/2A cells were routinely cultured in estrogen-free media. The day following seeding, the cells were treated with and without $10^{-9}$ M $E_2$ for 2 h. Twenty 15 cm plates of each cell line were harvested using a non-denaturing lysis buffer containing protease and phosphatase inhibitors. The lysates were syringed, debris was pelleted by centrifugation, and cleared lysates were transferred to coded vials and shipped to Georgetown University for proteomic analsis. At least 20 mg of protein per cell line per treatment group was collected.

### Experiments 4 and 5: Production of RNA samples for microarray analysis under Task 4

During year 1, we had produced RNA samples of MCF-7/WS8 and MCF-7/5C cells treated plus/minus $10^{-9}$M $E_2$ over a 96 h time course with time points corresponding to 2 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h. We collected 6 replicate samples per time point, isolated the RNA, and quality controlled the samples using a combination of electrophoresis and real-time PCR assays, and quality controlled the physiology of cells using DNA-based growth assays. During year 2, we have hybridized these MCF-7/WS8 and MCF-7/5C RNA samples to Agilent 4x44k human oligonucleotide microarrays and conducted preliminary bioinformatic analyses as described under Task 4 (Cunliffe). To complete production of RNA samples of the *in vitro* models during year 2, we have generated RNA samples of MCF-7/2A cells treated plus/minus $10^{-9}$ M $E_2$. However, since the MCF-7/2A cells undergo $E_2$-induced apoptosis with slower kinetics than the MCF-7/5C cells, it was necessary to treat the MCF-7/2A cells with $E_2$ over the same relatively short 96 h time course as used for the other two cell lines, but also treat the MCF-7/2A cells with $E_2$ over a relatively long time course extending from 3 to 9 days with harvesting of cells daily.

*Experiment 4)* (Short-term time course) Production of MCF-7/2A RNA samples for microarrays over a relatively short time course 96 h.

*Experiment 5)* (Long-term time course) Production of MCF-7/2A RNA samples for microarrays over relatively long time course of 3 to 9 days.

The protocols for producing MCF-7/2A RNA samples for the short-term and long-term time course microarray studies were very similar, and only differed in the number of cells seeded per plate and the time points. MCF-7/2A cells were seeded at 4 million and 0.6 million cells per plate in 15-cm plates in the short- and long-term time course microarray experiments, respectively. In the short-term experiment, the time points were the same as had been used to generate MCF-7/WS8 and MCF-7/5C RNA samples, namely 7 time points corresponding to 2 h, 6 h, 12 h, 24 h, 48 h, 72, and 96 h. In the long-term experiment, we used 7 time points corresponding to 3 days (72 h) through 9 days, with harvesting of samples every 24 h. In both experiment, 6 replicate 15-cm plates were used per treatment group per time point, and treatments were 10-9 M $E_2$ or 0.1% ethanol (vehicle) in estrogen-free RPMI-1640 plus 10% DCC-FBS. Media was replenished every 48 h. Cells were harvested in TRIzol reagent (Invitrogen; Carlsbad, CA). A total of 84 samples per time course, for a total of 168 samples, were collected. TRIzol lysates were coded and shipped to TGen for RNA purification.

**Growth response quality control**
In parallel with both the short-term and long-term time course microarray experiments, growth of the MCF-7/2A cells was assessed to ensure the cells showed the expected growth inhibition response to $E_2$ (Figure 2:1). MCF-7/2A cells were seeded at 40,000 cells per well in 6-well plates. The cells were incubated with control media, $10^{-9}$ M $E_2$, or $10^{-6}$ M fulvestrant media using 4 replicate wells per treatment. Cells were allowed to grow for 9 days, and media was replenished every other day on days 2, 4, 6 and 8. On the last day, cells were washed with ice-cold phosphate-buffered saline and frozen. Lysates were generated by sonicating the frozen cells in hypotonic (0.1X) Hank's balanced salt solution. Cellular DNA content in the lysates were measured using the DNA Quantitation kit (BioRad) based on the fluorescent dye Hoechst 33258 and compared to a standard curve of known calf thymus DNA amounts by linear regression analysis. We observed that $E_2$ inhibited growth of MCF-7/2A cells by 54% and 71%, in the short- and long-term time course microarray experiments, respectively, compared to control-treated cells (both *P*-values < 0.001) over a 9 day period. Therefore, the MCF-7/2A cells exhibited the expected growth inhibition to $E_2$ treatment **(Figure 6)**.
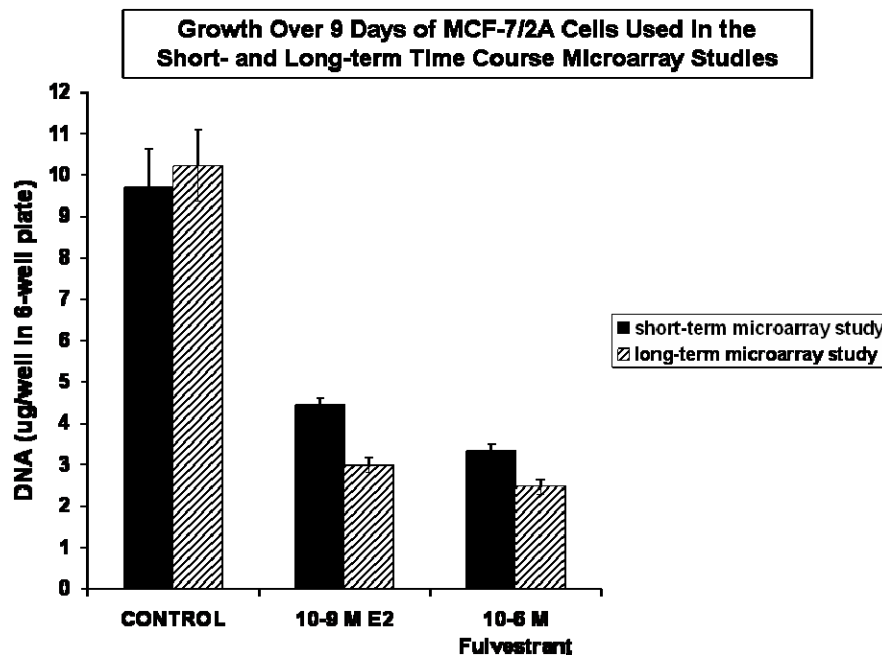
**Figure 6. Growth of MCF-7/2A Cells over 9 Days.** These cells will be used in the short-term and long-term time course microarray studies.

## RNA Purification and RNA Integrity Quality Control

At TGen, TRIzol lysates were heated to 65° C for 30 minutes, and extracted with chloroform to form an aqueous phase solution, which was mixed 1:1 by volume with 80% ethanol. The resulting mixture was applied to RNeasy (Qiagen) anion-exchange columns and processed following the manufacturer's directions to elute purified total RNA. The purified RNA samples have been quality controlled for RNA integrity by electrophoresis and assessing the ribosomal 28S and 18S rRNA bands using an Agilent Lab-on-a-Chip Bioanalyzer. An example of the elecrophoresis quality control for RNA integrity is shown in **Figure 7**.

## RNA Expression Quality Control

TGen has shipped the purified MCF-7/2A RNA samples back to FCCC for further quality control analysis using real-time PCR assays. PUM1 mRNA levels were measured as an endogenous normalization gene and as an indicator of overall RNA quality (5). pS2 mRNA levels were measured as an indicator of $E_2$-stimulated gene expression for samples treated with $E_2$ for $\geq$ 24 h. c-Myc mRNA levels were only measured in short-term time course samples, as c-Myc is an early response gene, and appropriate for assessing $E_2$-stimulated growth for samples treated with E2 for 2 h to 12 h.

Single-strand cDNA was synthesized from RNA using random hexamers and oligodeoxynucleotide $dT_{15}$ as primers and an MuLV reverse transcriptase-based kit (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). Real-time PCR assays were carried out using 10 ng cDNA per well in a total volume of 25 ul and either the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) for dual fluorescently-labeled probe-based assays, or Power SYBR Green Master Mix (Applied Biosystems) for non-probe-based assays. Each cDNA sample was assayed in triplicate. PCR product accumulation was measured in real-time using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). To quantitate RNA levels, the threshold cycles of PCR product accumulation of unknown samples were compared against a standard curve consisting of 6 2-fold serial dilutions of reference MCF-7/WS8 cDNA. RNA levels of the target gene were normalized to either PUM1 RNA levels. PCR primer sequences were as follows: PUM1 forward 5'-AAT GCA GGC GCG AGA AAT-3', PUM1 reverse 5'-TTG TGC AGC TGA GGA ACT AAT GA-3, PUM1 probe 5'-[6FAM]-CCT GTT CGA CTT GTA GCT CCT GCC CC-[BHQ1]-3'; c-myc forward 5'-GCC ACG TCT CCA CAC ATC AG-3', c-Myc reverse 5'-TCT TGG CAG CAG GAT AGT CCT T-3', c-Myc probe 5'-
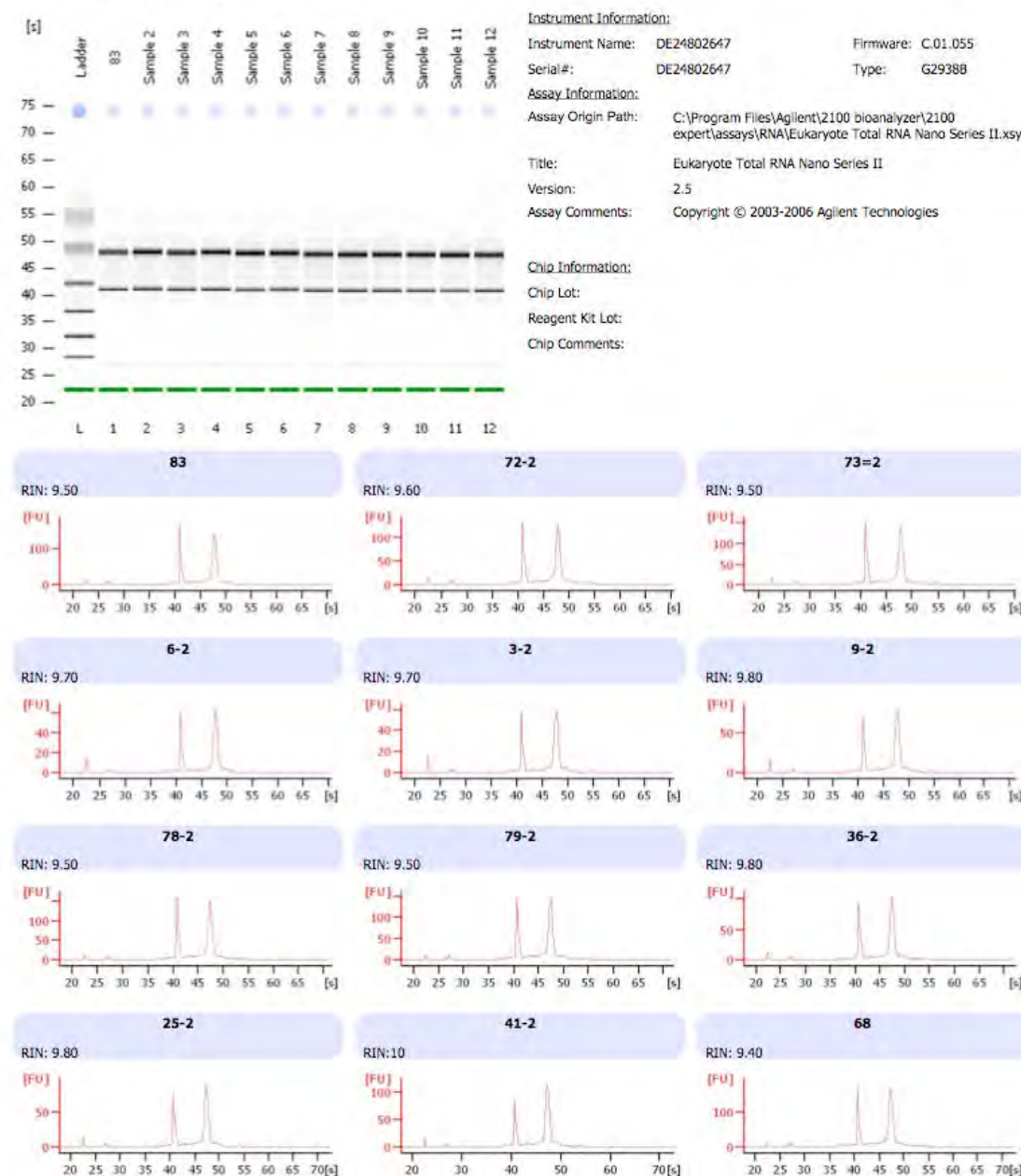
[6FAM]-ACG CAG CGC CTC CCT CCA CTC-[BHQ1]-3'; pS2 forward 5'-CAT CGA CGT CCC TCC AGA AGA G-3', pS2 reverse 5'-CTC TGG GAC TAA TCA CCG TGC TG-3'.

The quality control qRT-PCR data for PUM1, c-Myc, and pS2 mRNA levels in the 96 h short-term MCF-7/2A time course are shown in **Figures 8, 9, and 10**, respectively. Similarly, the quality control qRT-PCR data for PUM1 and pS2 mRNA levels in the 4 day long-term MCF-7/2A time course are shown in **Figures 11 and 12**, respectively. These results indicated that only 3 samples out of 168 samples from both time courses combined do not pass qRT-PCR quality control. The following samples will not be used in further gene expression microarray hybridizations and analysis:1) one sample in the short time course (12 h Control treatment, Replicate 2) contains low quality RNA as shown by a low PUM1 mRNA level **(Figures 8)**, this low level of PUM1 in the short time course resulted in aberrantly high pS2/PUM1 expression in the same sample (**Figures 10)**; and 2) two samples in the short time course (2 h Control, Replicate 4  and 2 h E$_2$, Replicate 4) show an inverse pattern relative to that expected of c-Myc expression **(Figures 9)**. All the remaining samples showed the expected patterns of expression.

These remaining 165 MCF-7/2A short and long-term time course experiments are fully quality controlled for growth of the cells, RNA integrity, and gene expression indicators, and will be hybridized to Agilent 4x44k human oligonucleotide chips as has been carried out with the MCF-7/WS8 and MCF-7/5C samples described under Task 4 (Cunliffe).

Assay Class:    EukaryoteTotal RNA Nano
Data Path:      C:\..._EukaryoteTotal RNA Nano_DE24802647_2008-07-22_12-32-30.xad

Created:   7/22/2008 12:32:30 PM
Modified:  7/22/2008 12:55:44 PM

**Electrophoresis File Run Summary**

Instrument Information:

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| Serial#: | DE24802647 | Type: | G2938B |

Assay Information:

Assay Origin Path:  C:\Program Files\Agilent\2100 bioanalyzer\2100 expert\assays\RNA\Eukaryote Total RNA Nano Series II.xsy

Title:          Eukaryote Total RNA Nano Series II

Version:        2.5

Assay Comments: Copyright © 2003-2006 Agilent Technologies

Chip Information:

Chip Lot:

Reagent Kit Lot:

Chip Comments:



2100 expert (B.02.03.SI307)          © Copyright 2003-2006 Agilent Technologies, Inc.          Printed:   7/22/2008 1:01:29 PM

**Figure 7.  RNA microfluidic electrophoresis using the Agilent Bioanalyser.** Shown are representative results from analysis of 12 samples from the MCF-7:2A 96h time course.  All samples shown have a RIN number of >9.0.  Synthesis of cRNA probes for microarray hybridization is recommended for RNA of RIN quality >8.0.  A total of 3 RNAs from 168 isolated failed our RIN QC, meaning there will be 5 instead of 6 replicate microarrays.
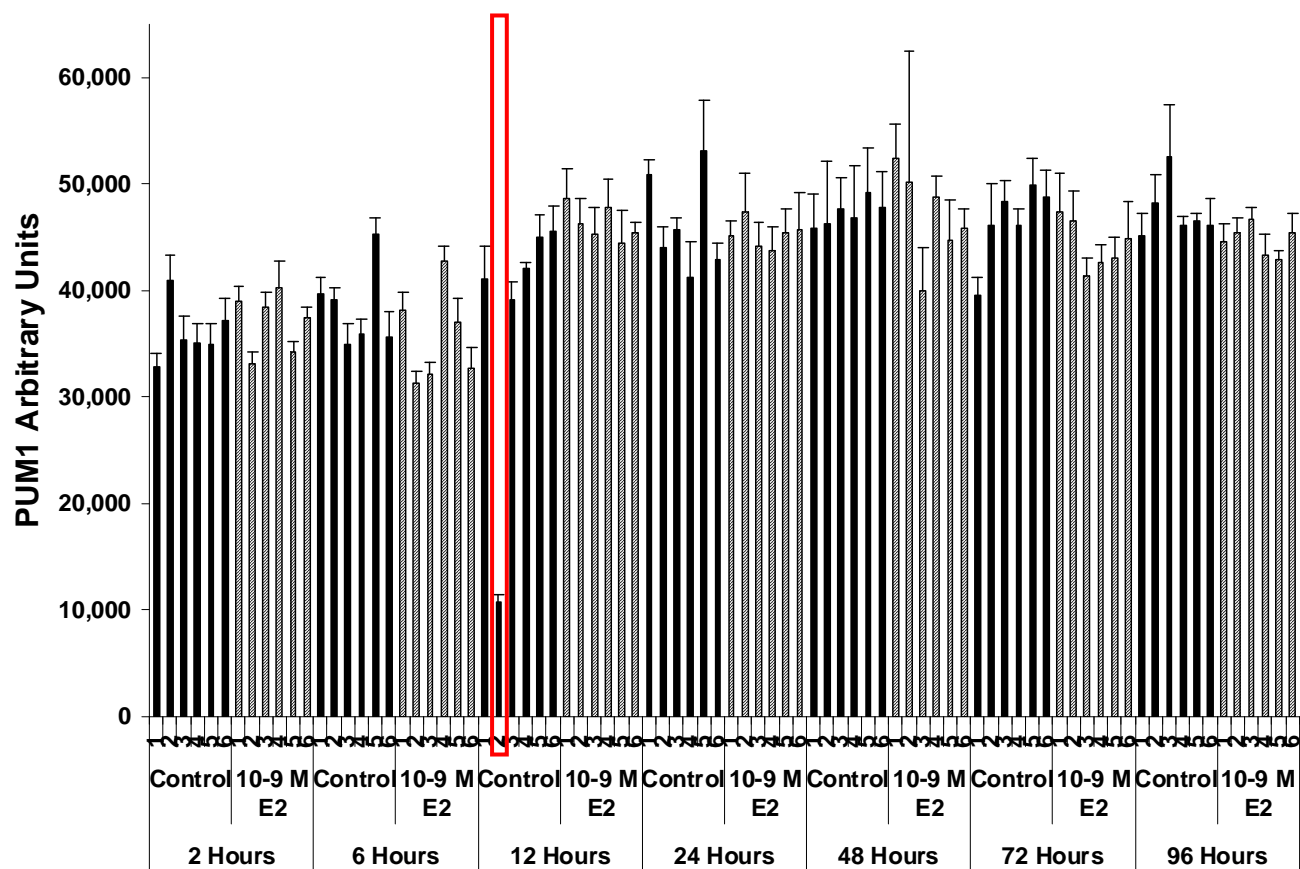
14

**Figure 8. PUM1 mRNA levels in MCF-7/2A RNA Samples from the 96 h Short-term Time Course to be Used for Microarray Analysis.** The one sample that does not pass quality control is indicated by a red box (12 h Control treatment, Replicate 2).
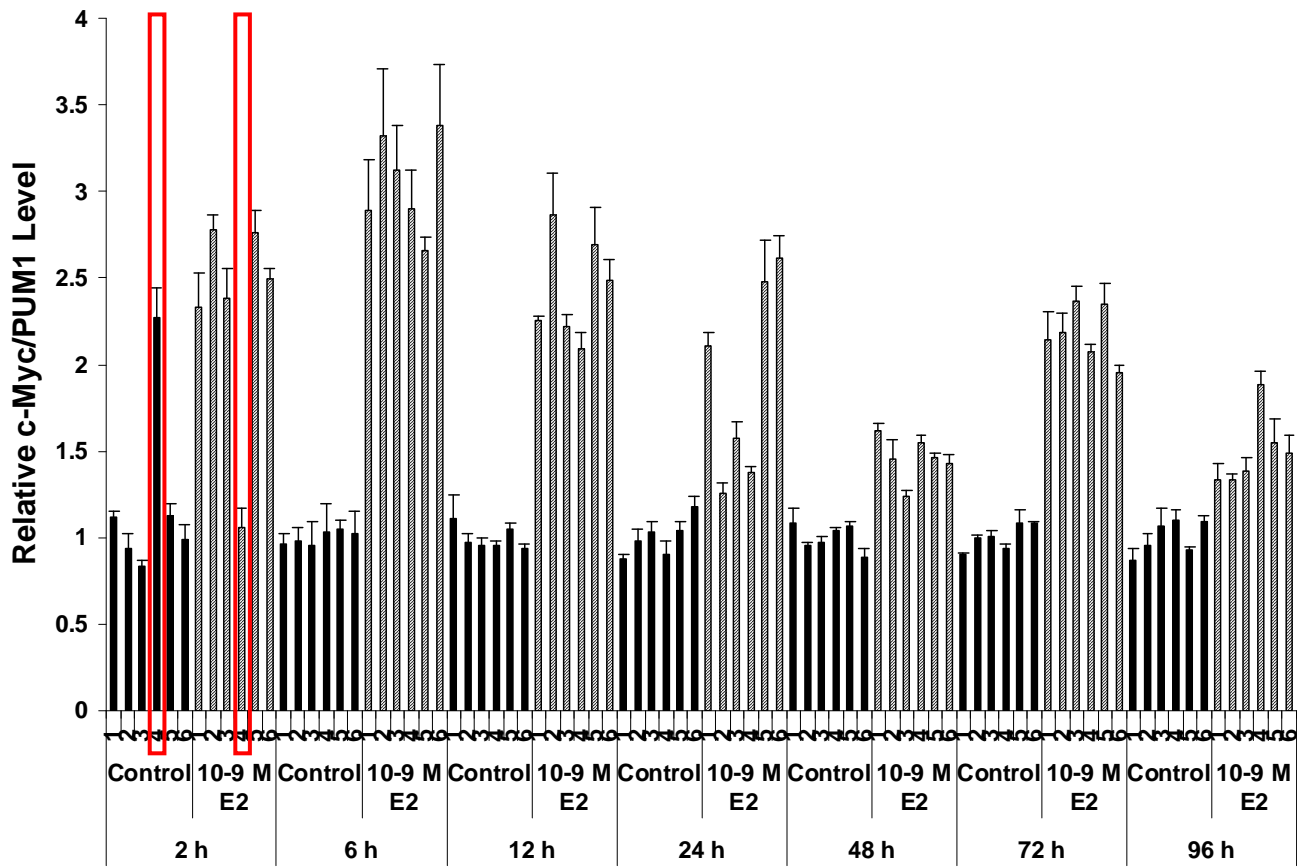
**Figure 9. c-Myc mRNA levels in MCF-7/2A RNA Samples from the 96 h Short-term Time Course to be Used for Microarray Analysis.** The two samples that do not pass quality control are indicated by a red box (2 h Control treatment, Replicate 4; and 2 h E2 treatment, Replicate 4).
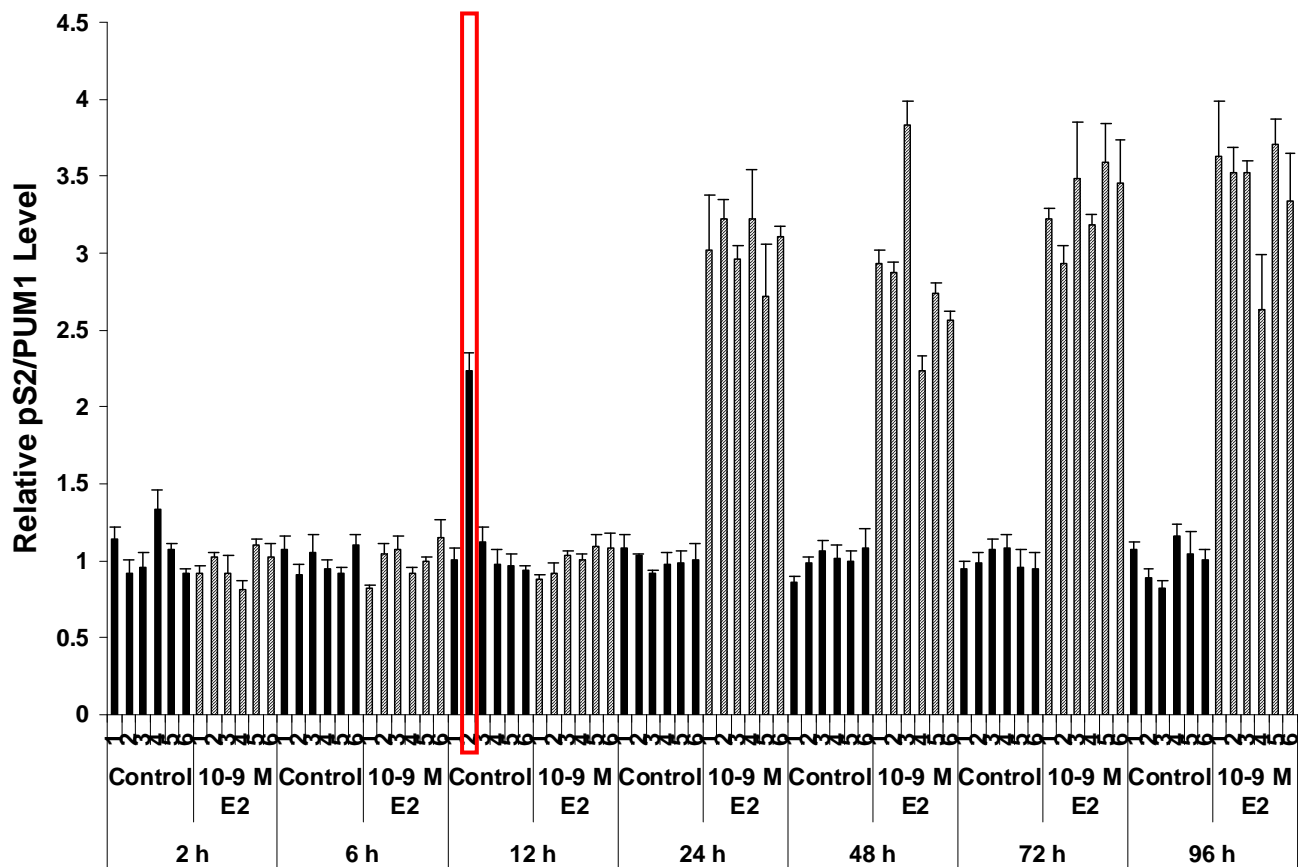
**Figure 10. pS2 mRNA levels in MCF-7/2A RNA Samples from the 96 h Short-term Time Course to be Used for Microarray Analysis.** The one sample that does not pass quality control is indicated by a red box (2 h Control treatment, Replicate 4; and 2 h E2 treatment, Replicate 4).
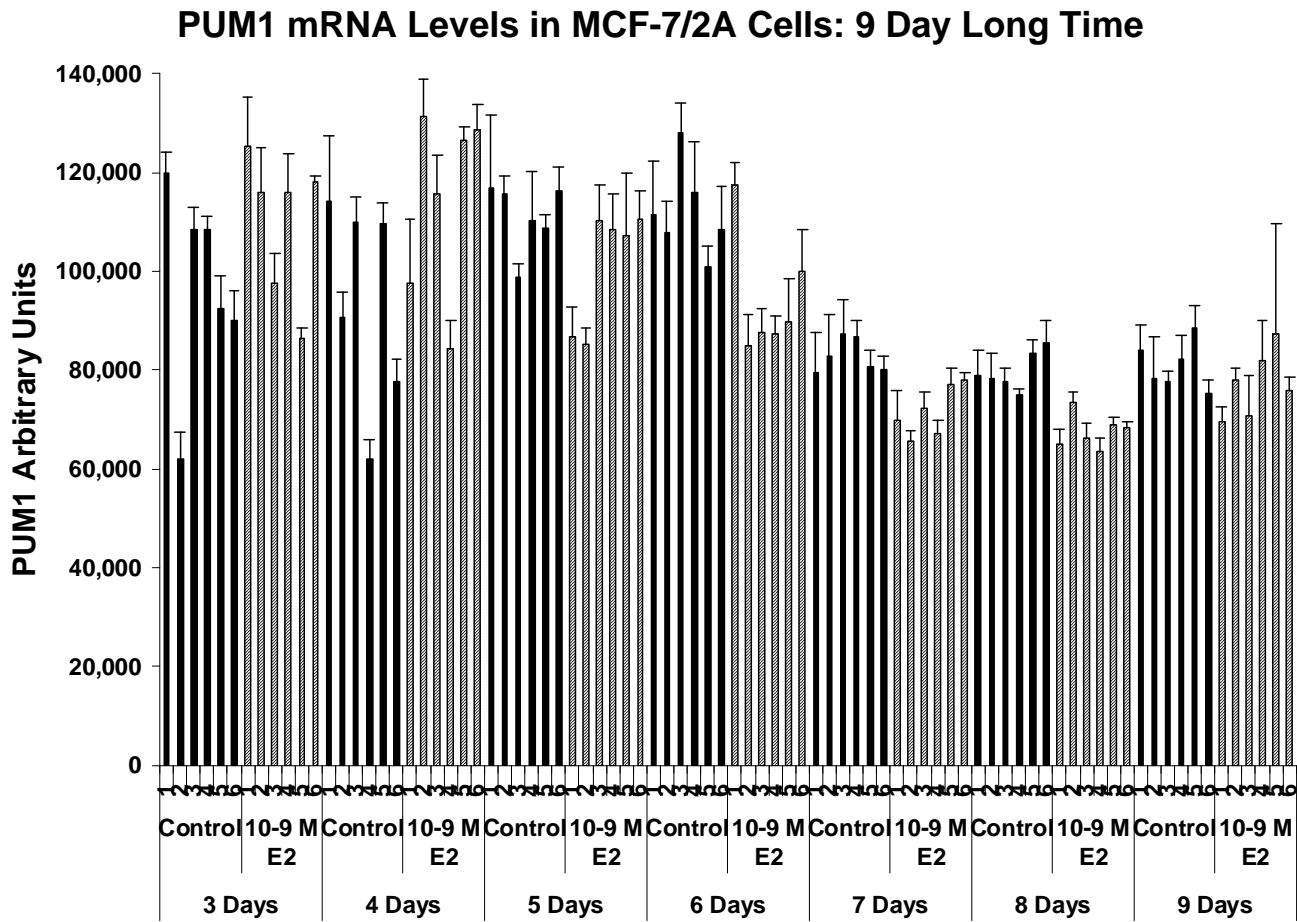
**Figure 11. PUM1 mRNA levels in MCF-7/2A RNA Samples from the 4 day Long-term Time Course to be Used for Microarray Analysis.** All samples passed PUM1 expression quality control.

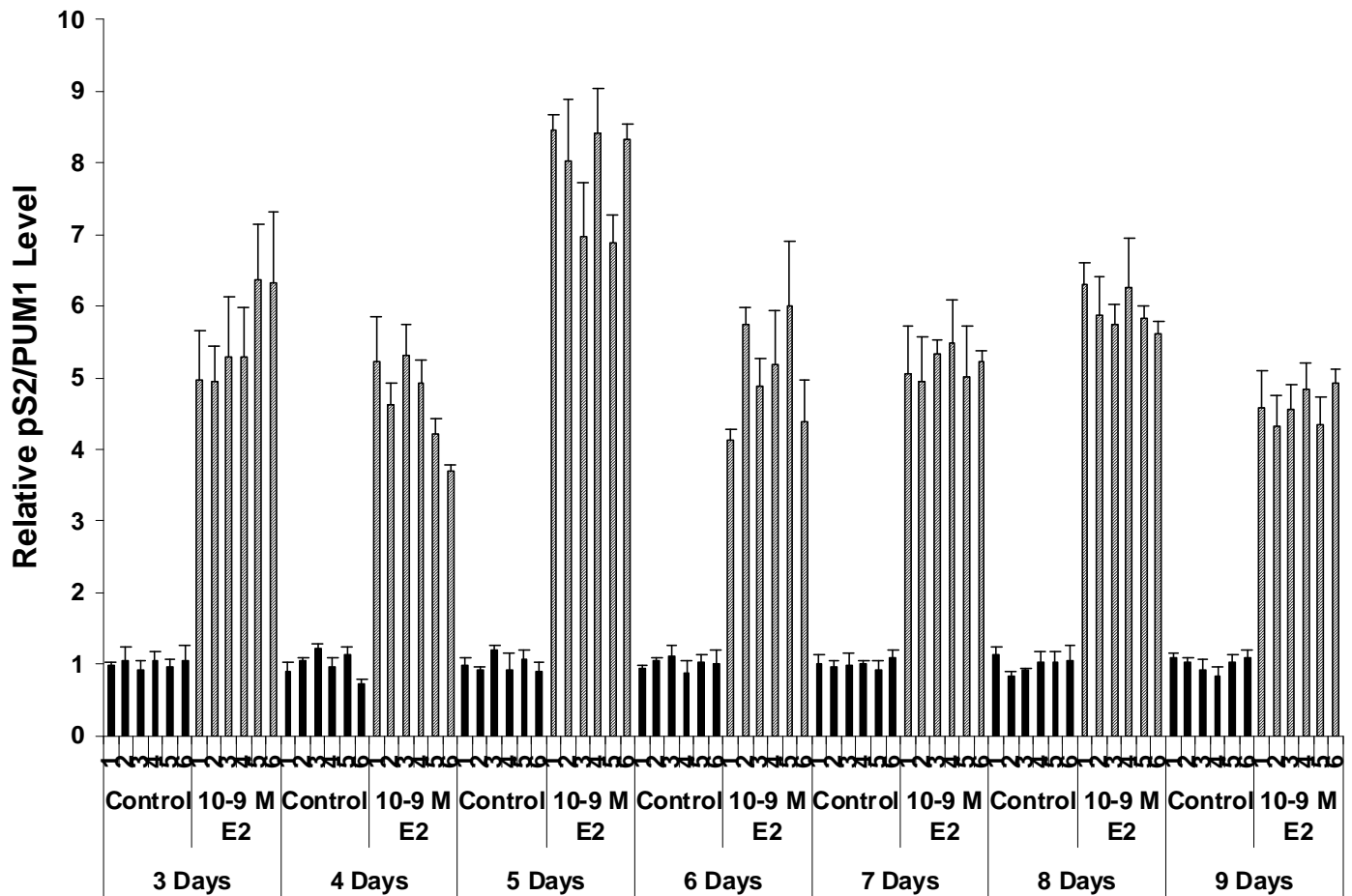**pS2 mRNA Levels in MCF-7/2A Cells: 9 Day Long Time Course**

**Figure 12.  pS2 mRNA levels in MCF-7/2A RNA Samples from the 4 day Long-term Time Course to be Used for Microarray Analysis.** All samples passed pS2 expression quality control.

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Pink JJ, Jiang SY, Fritsch M, Jordan VC.  An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. *Cancer Res* 1995: 55(12):2583-90.

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**TASK 2b - FCCC/Jordan, Lewis-Wambi - Studies carried out by Dr. Joan Lewis-Wambi in the Jordan laboratory at FCCC**

**Task 2b (FCCC/ Jordan, Lewis-Wambi): Confirm and validate developing pathways of $E_2$-induced breast cancer cell survival and apoptosis.**

Here we report work completed on Tasks 2b at the Fox Chase Cancer Center site during year 2 of this COE involving characterization the kinetics of $E_2$-induced apoptosis in MCF-7:2A and MCF-7:5C cells, and the sensitization to apoptosis in MCF-7:2A cells using L-buthionine sulfoximine (BSO).

**KINETICS OF $E_2$-INDUCED APOPTOSIS IN LONG TERM ESTROGEN-DEPRIVED MCF-7:2A AND MCF-7:5C CELLS, AND THE ROLE OF GLUTATHIONE SUPPRESSION IN SENSITIZING ANTIHORMONE RESISTANT CELLS TO $E_2$-INDUCED APOPTOSIS**

To address Task 2b, our laboratory has created a panel of MCF-7 breast cancer cells *in vitro* that have been $E_2$-deprived (ED) for several years to replicate resistance to estrogen deprivation (i.e. resistance to aromatase inhibitors). The parental (naïve) MCF-7 cell line responds to physiologic concentrations of $E_2$ with growth. The two estrogen-deprived breast cancer cell clones, MCF-7:5C and MCF-7:2A cells, both of undergo apoptosis (programmed cell death) in the presence of $E_2$ **(Figure 13)**. Interestingly, it appears that the apoptotic effect of $E_2$ in MCF-7:2A cells occurs after 7 days of treatment whereas in MCF-7:5C cells apoptosis occurs after 2 days of $E_2$ treatment **(Figures 13, 14)**.
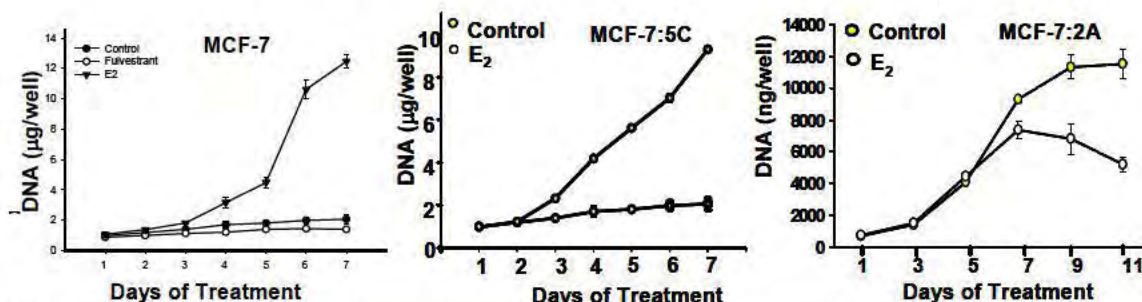


**Figure 13. Effects of $E_2$ on the growth of parental MCF-7 cells and long-term $E_2$-deprived MCF-7:5C and MCF-7:2A cells.** For growth assays, approximately $2 \times 10^4$ MCF-7, MCF-7:2A, and MCF-7:5C cells were seeded in 24-well plates in estrogen-free RPMI medium and then treated with either $E_2$ ($10^9$ M) or fulvestrant ($10^6$ M) for 7 or 11 days. Note, for fulvestrant treatment of MCF-7 cells, growth assay was performed in phenol-red RPMI medium containing 10% fetal bovine serum (FBS). Cells were harvested at the indicated time point and total DNA (µg/well or ng/well) was determined using a DNA fluorescence quantitation kit. Data shown is representative of four separate experiments with similar results and arrow bars indicate mean ±SE of triplicate values.



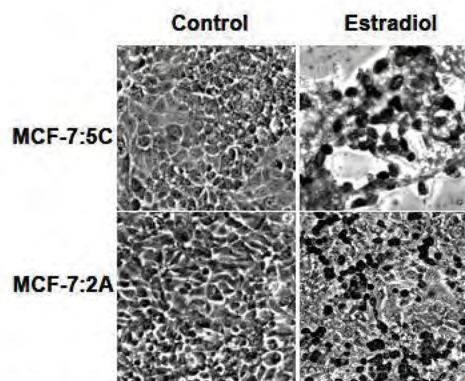**Figure 14. $E_2$ induces apoptosis in MCF-7:5C and MCF-7:2A cells.** For apoptosis determination, MCF-7:5C and MCF-7:2A cells ($2 \times 10^5$) were seeded in 8-well chamber slides in estrogen-free RPMI medium and then treated with $10^9$

M $E_2$ or ethanol vehicle (control) for 3 days (MCF-7:5C cells) or 7 days (MCF-7:2A cells). Apoptotic cells were identified by TdT-mediated dUTP nick end labeling (TUNEL) using the In Situ Cell Death Detection Kit, POD (Roche-Applied Science, cat# 11684817910). The In Situ Cell Death Detection Kit is designed as a precise, fast and simple, non-radioactive technique to detect and quantify apoptotic cell death at single cell level in cells and tissues. **Apoptotic cells are darkly stained**. Images were viewed and captured by an inverted Nikon TE300 objective microscope equipped with a Spot RT (Diagnostic Instruments) monochrome camera.

**Glutathione suppression enhances the apoptotic effect of $E_2$ in estrogen-deprived MCF-7:2A breast cancer cells.**

Interestingly, during the process of our studies, we serendipitously found a synthetic compound called L-buthionine sulfoximine (BSO) which has the ability to enhance the apoptotic effect of $E_2$ in MCF-7:2A cells. BSO is a potent inhibitor of glutathione synthesis (1). Glutathione is a naturally occurring reducing agent (i.e. antioxidant) that protects cells from oxidative stress and hence apoptosis (2). Previous studies have shown that GSH depletion with BSO leads to cell death and highly sensitizes tumor cells to apoptosis (programmed cell death) induced by standard chemotherapeutic agents (3-7). We found that pretreatment of MCF-7:2A cells with BSO caused these cells to undergo apoptosis in the presence of $E_2$ **(Figure 15B, 15C)**. Exposure of MCF-7:2A cells to 1 n$M$ $E_2$ or 100 µ$M$ BSO for 72 hours did not produce apoptosis, however, the combination treatment produced a 7-fold increase in apoptosis which was assessed by Annexin V staining.
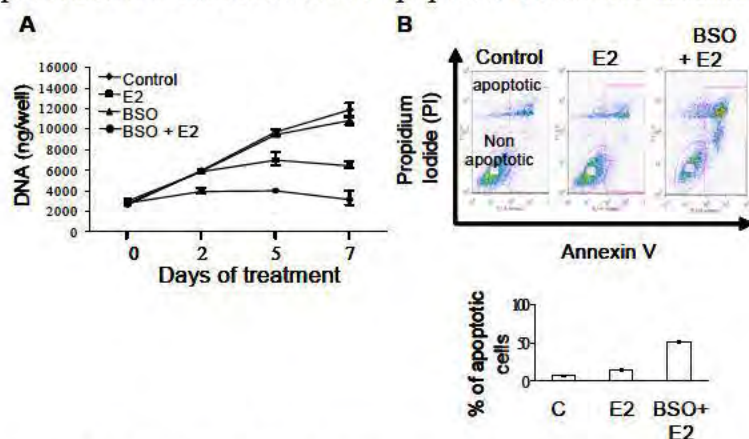


**Figure 15.** BSO plus $E_2$ induces apoptosis of aromatase inhibitor-resistant. *A,* MCF-7:2A cells were treated with 1 nM $E_2$, 100 µM BSO, 100 µM BSO + 1 nM $E_2$, or nothing (control) for 7 days and cells were harvested at the indicated time points and total DNA (ng/well) was quantitated using a DNA quantitation kit. *B,* Annexin V staining for apoptosis in MCF-7:2A cells following MCF-7:2A cells 72 hours of treatment with the indicated drugs. Cells were then stained with FITC- annexin V and propidium iodide (PI) and analyzed by flow cytometry. Representative cytograms are shown for each group. Quantitation of apoptosis (percent of control) in the different treatment groups is shown on the *right panel.*

We also found that MCF-7:2A cells possessed elevated levels of glutathione compared to parental MCF-7 cells **(Figure 16A)** and these cells expressed significantly elevated levels of glutathione synthesis enzymes as determined by and real-time PCR analysis **(Figure 16B)**.

**A**
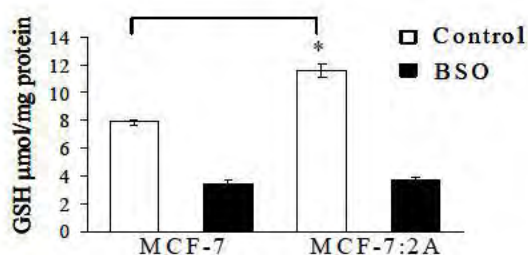


**B**



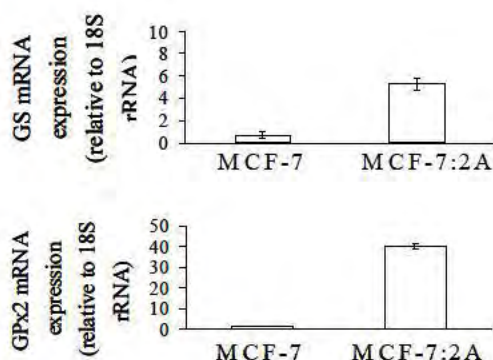**Figure 16.** Intracellular glutathione levels in parental MCF-7 cells and hormone-independent MCF-7:2A breast cancer cells. **A,** parental MCF-7 cells and MCF-7:2A cells were seeded at $2 \times 10^6$ cells per 100 mm culture plates in estrogenized media (phenol red-replete RPMI media containing 10% FBS) and estrogen-free (phenol red-free RPMI media containing 10% dextran coated charcoal-treated FBS), respectively, and after 24 hours were treated with nothing (control) (*white columns*) or 100 mM BSO (*black columns*) for 24 hours. Total cellular glutathione was measured using a Glutathione Colorimetric microplate assay kit. *Columns,* mean from three separate experiments; *bars,* SE. *, $P < .005$, with respect to parental MCF-7 control. **B,** quantitative real-time PCR of glutathione sythetase (GS) (*top left*) and glutathione peroxidase 2 (GPx2) (*bottom left*) mRNA expression in MCF-7 and MCF-7:2A cells.

## Mechanism by which BSO enhances the apoptotic effect of $E_2$ in MCF-7:2A cells involves Bcl-2 and the mitochondrial pathway.

It has been reported that the action of Bcl-2 is related to antioxidant protection against cellular damage. Previous studies have shown that Bcl-2 overexpression raises cellular glutathione levels (8). We therefore examined whether the apoptotic effect of BSO plus $E_2$ in MCF-7:2A cells involves Bcl-2. Western blot analysis were performed on cells treated with $E_2$ alone, BSO alone, or $E_2$ + BSO for 24, 48, and 72 hours and Bcl-2, phosphorylated Bcl-2, and Bcl-xl protein were measured. **Figure 17A** shows that $E_2$ + BSO significantly reduced phosphorylated Bcl-2 and Bcl-xl protein in both MCF-7:2A and MCF-7:5C cells in a time-dependent manner, however, Bcl-2 protein level did not significantly change with the combination treatment. It will be interesting to see whether suppression of Bcl-2 or Bcl-xl expression using small interfering RNA (siRNA) has the ability to enhance the apoptotic effects of $E_2$ alone or $E_2$ plus BSO. These experiments are currently being performed in our laboratory. Induction of apoptosis by the combination treatment of $E_2$ plus BSO was also evidenced by changes in cytochrome *c* release and activation of caspase-7 and poly(ADP-ribose) polymerase (PARP) (**Figure 17B, 17C**), events which are all associated with mitochondrial-mediated apoptosis.
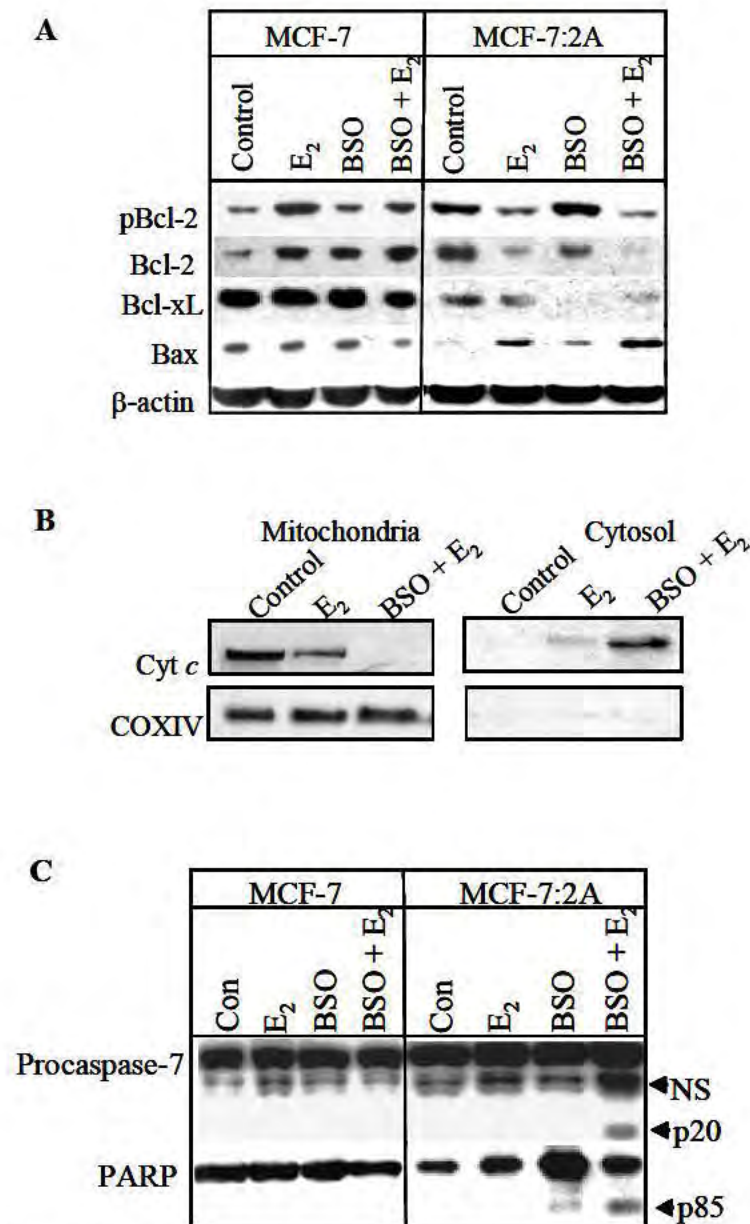
**Figure 17.** Effect of BSO and $E_2$ on Bcl-2 family protein expression and mitochondrial function in MCF-7 and MCF-7:2A cells. **A,** western blot analysis for pBcl-2, Bcl-2, Bcl-$x_L$, and Bax protein expression in parental MCF-7 cells and MCF-7:2A cells following 48 hours of treatment with ethanol vehicle (Control), 1 nM $E_2$, 100 μM BSO, or $E_2$ + BSO. Equal loading was confirmed by reprobing with an antibody against β-actin. **B,** cytochrome $c$ release from the mitochondria to the cytosol after treatment with $E_2$ alone or BSO and $E_2$ for 48 hours was determined as described in Materials and methods. Anti-COXIV (subunit IV) antibody was used as a control to demonstrate that mitochondrial protein fractionation was successfully achieved. **C,** cleavage of caspase 7 and PARP (72 hours) was assessed by Western blot using specific antibodies. The upper band of caspase 7 represents the full length protein and the lower band (p20, arrow) represents the cleaved activated product; NS, nonspecific. Full length PARP is approximately 116 kDa; cleaved (active) PARP is 85 kDa (arrow). The results are representative of three independent experiments.

### BSO inhibits the growth of MCF-7:2A cells *in vivo.*

To determine whether the effect of BSO plus $E_2$ was relevant *in vivo*, we used a xenograft model in which MCF-7:2A cells were injected into athymic mice (n=20). After 20 days postinjection, tumors grew to a mean crossectional area of 0.30 cm$^2$ and mice were randomized to four groups; placebo (saline), $E_2$, BSO, or the combination of BSO plus $E_2$, as described in a materials and methods. After 7 days of treatment, tumor growth was reduced by 25% in mice treated with $E_2$ alone whereas in the BSO and BSO plus $E_2$ group tumor growth was reduced by 40% and 60%, respectively, compared to the placebo group which showed a 7% increase in

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growth (**Figure 18A**). Interestingly, we found that BSO *in vitro* had a relatively small effect on growth, however, *in vivo* its effect was very pronounced, thus suggesting the possibility of altered glutathione metabolism *in vivo*. We performed histology on tumors taken from placebo, $E_2$, BSO, and BSO plus $E_2$ groups at day 27. H&E staining of the BSO plus $E_2$-treated tumors revealed less tumor cells and more intercellular matrix, significantly less mitoses, chromatin clumping and dark staining which are associated with apoptosis, and enhanced abnormalities in shape and size, compared to tumors from placebo or BSO or $E_2$-treated groups (**Figure 18B**). We also characterized the proliferative status of these cells by staining tumors for the expression of Ki67, a marker of cell proliferation. We observed a 32% decrease ($P < 0.001$) in the number of Ki67 stained tumors from the BSO plus $E_2$-treated group and a 21% decrease in the BSO-treated group compared to the placebo group whereas $E_2$ treatment caused an 8% increase in Ki67 staining (**Figure 18C**). Overall, these data show that BSO either alone or in combination with $E_2$, reduces tumor growth by possibly increasing apoptosis and decreasing the proliferation of tumor cells.
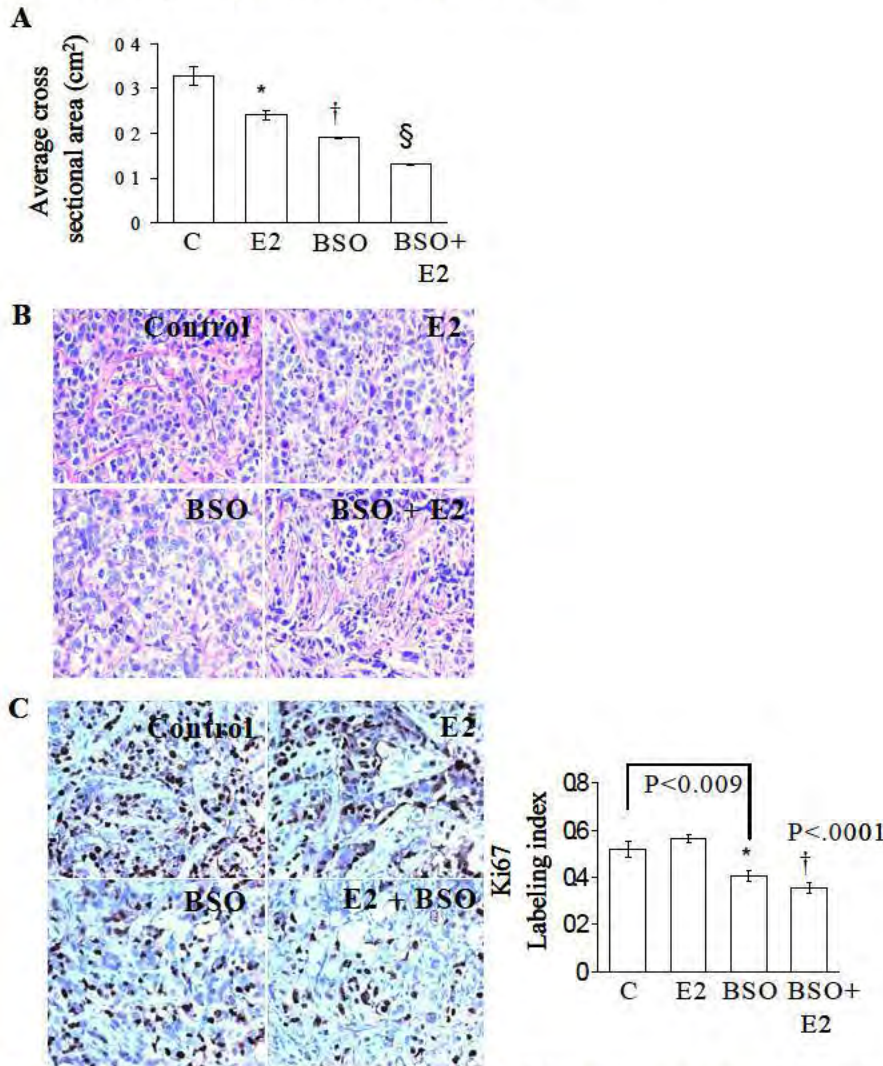


**Figure 18.** BSO inhibits the growth of MCF-7:2A tumors *in vivo*. 4-5 weeks athymic nude mice (n = 20) were injected with MCF-7:2A breast cancer cells and after 20 days when tumors had reached a mean cross sectional area of 0.3 cm$^2$, animals were randomized into 4 groups and were treated with placebo (saline), $E_2$, BSO, or BSO plus $E_2$ for 7 days as described in materials and methods. BSO (4 mmol/kg weight) was diluted in saline and was injected i.p. daily. **A,** tumor size was measured everyday and cross-sectional area was calculated by multiplying the length (*l*) by the width (*w*) by $\pi$ and dividing the product by four (i.e., $lw\pi/4$). Data is shown as mean ± SE. *, $P < 0.05$, control group compared with $E_2$ group; †, $P < 0.002$ control group compared with BSO group; §, $P < 0.001$ control group compared with BSO+$E_2$ group. **B**, microscopy of H&E-stained histological sections of MCF-7:2A tumors treated with placebo, $E_2$, BSO, and BSO in combination with $E_2$. **C**, immunohistochemical analysis of the proliferation marker Ki-67 in MCF-7:2A tumors treated with placebo, $E_2$, BSO, or BSO plus $E_2$. Three to four tumors per treatment group were analyzed.

Overall, our findings indicate that glutathione participates in retarding apoptosis in hormone-resistant human breast cancer cells such as MCF-7:2A and that depletion of this molecule may be critical in predisposing resistant cells to apoptotic cell death. **This work has recently been submitted to Breast Cancer Research and is under review.** A copy of this manuscript is included in the appendix.

**Long-term estrogen deprivation enhances the migratory and invasive potential of breast cancer cells.**
Invasion and metastasis are the hallmarks of cancer malignancy and they are the primary cause of patient mortality during breast cancer progression. Invasion refers to the ability of cancer cells to penetrate through the membranes that separate them from healthy tissues and blood vessels and metastasis refers to the spreading of cancer cells to other parts of the body. In order for a transformed cell to metastasize, it must first lose adhesion, penetrate and invade the surrounding extracellular matrix (ECM), enter the vascular system, and adhere to distant organs. These processes require extensive alterations in gene expression profiles, including the down-regulation of genes involved in cell anchorage and the up-regulation of genes involved in cell motility and matrix degradation. Using expression array analysis which was confirmed by real-time PCR and Western blot analyses, we discovered that a novel gene called carcinocinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6). CEACAM6 is an intercellular adhesion molecule (9) that is overexpressed in a wide variety of human cancers, including colon, breast, and lung (10, 11) and is associated with tumourigenesis, tumour cell adhesion, invasion and metastasis (12-14) and antihormone resistance (15, 16). Specifically, we found that CEACAM6 mRNA and protein was overexpressed in MCF-7:5C and MCF-7:2A cells, respectively, and that this overexpression was associated with a 6 to 15-fold increase in the invasive phenotype of these cells compared to parental MCF-7 cells which are non-invasive and express low levels of CEACAM6. We also found that suppression of CEACAM6 expression using small interfering RNA (siRNA) completely reversed the invasiveness of MCF-7:5C and MCF-7:2A cells. Overall, this finding establishes CEACAM6 as a unique mediator of migration and invasion of drug resistant estrogen deprived breast cancer cells and it suggests that this protein could be an important biomarker of metastasis. **This work has been accepted for publication in the European Journal of Cancer (2008).** A copy of this manuscript is included in the appendix.

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**TASK 2 - FCCC/Jordan - Studies carried out by Dr. Surojeet Sengupta in the Jordan laboratory at FCCC**

**Task 2b (FCCC, Jordan/Sengupta): To confirm and validate developing pathways of E$_2$-induced breast cancer cell survival and apoptosis.**

Here we report work completed on Tasks 2b at the Fox Chase Cancer Center site during year 2 of this COE involving the role of X-box binding protein 1 (XBP1) in E$_2$-induced breast cancer cell survival.

**ROLE OF X-BOX BINDING PROTEIN-1 (XBP1IN MODULATING ESTROGEN MEDIATED GROWTH OF BREAST AND ENDOMETRIAL CANCER CELLS BY REGULATING BCL-2 (B CELL LYMPHOMA-2).**

**Introduction**
X-box binding protein 1 (XBP1), is a known estrogen regulated gene which is highly co-expressed with estrogen receptor alpha (ERα) in breast cancer patients. Several DNA micro-array studies have found XBP1 gene regulated by estrogen in ER positive breast cancer cell lines and breast cancers. In addition, recruitment of ERα on the XBP1 promoter as well as enhancer regions has been detected by experiments using Chromatin immuno-precipitation (ChIP) followed by tiled microarray on human chromosomes 21 and 22. XBP1 is a transcription factor, identified as basic region leucine zipper (bZIP) belonging to the ATF/CREB family, known to be involved in unfolded protein response (UPR) where it activates a distinct set of genes and regulates endoplasmic reticulum stress mediated apoptosis.

**Estrogen Regulation of XBP1**
We studied the time course of estrogen regulation of XBP1 by the micro-array study of MCF-7: WS8 cells **(Figure 19)** and further confirmed it using quantitative real time PCR. XBP1 was found to be upregulated very early (within 2 hr) after estrogen treatment and remain elevated up to 48 hrs **(Figure 20A)**. The estrogen regulation of XBP1 was completely abrogated by fulvestrant (complete ER antagonist) and 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (transcriptional inhibitor) but not by Cycloheximide, (translational inhibitor) **(Figure 20B)**. These results clearly indicate that estrogen regulation of XBP1 is mediated by estrogen receptor and at the transcription level. Cycloheximide insensitivity suggested that it is a primary response, *i.e.*, *de novo* protein synthesis is not required for induction of XBP1 by estrogen. Very similar results were observed in ER positive endometrial cancer cells, ECC1 **(Figure 20C, 20D)** indicating consistent regulation of XBP1 by estrogen across different cell lines.
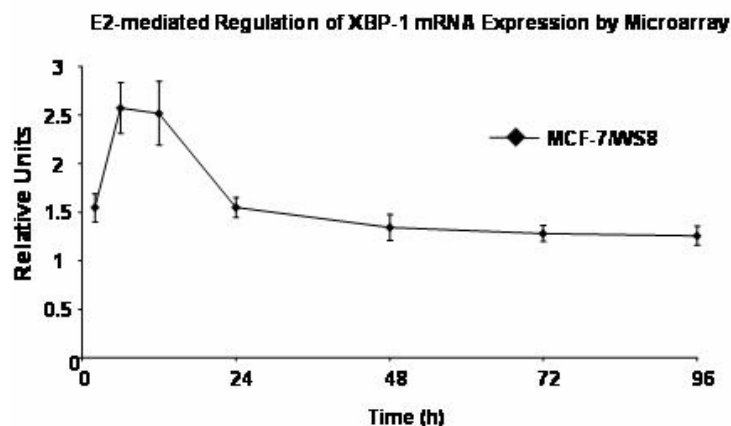


**Figure 19. Estrogen Regulation of XBP1 in MCF-7 cells measured by gene expression microarrays.** MCF-7 cells were treated with E$_2$ (1nM) over a 96 h time course. RNA was extracted and Agilent microarray studies were performed.
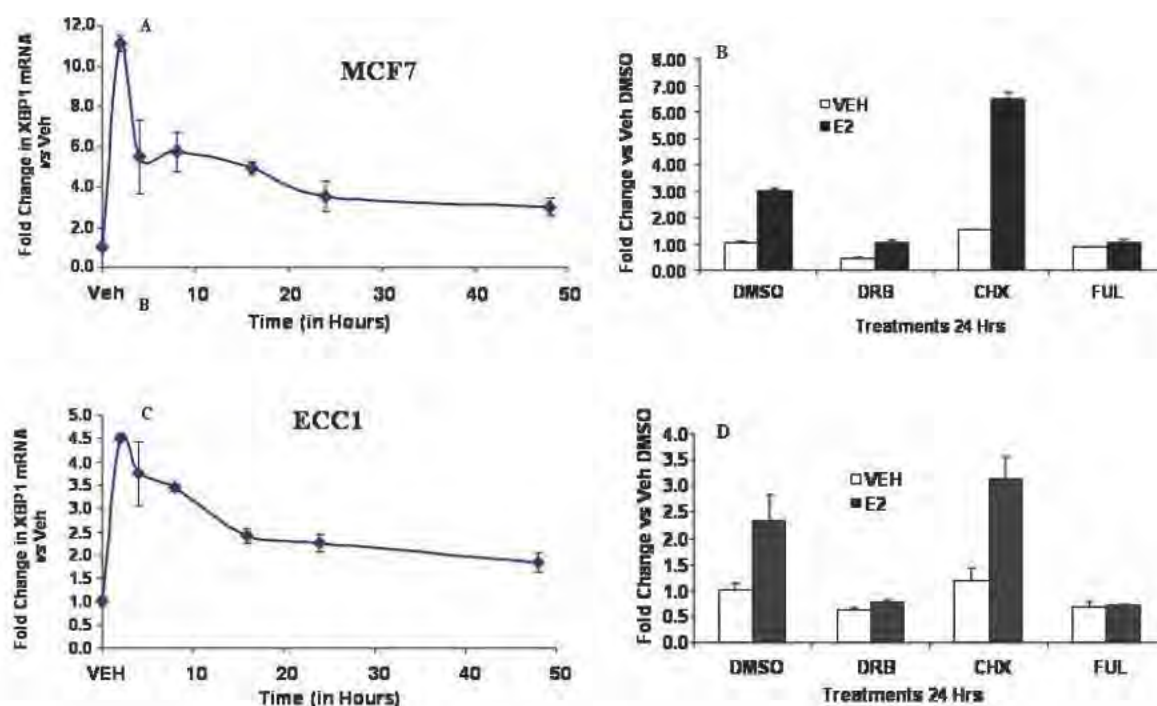
**Figure 20. Estrogen Regulation of XBP1 in MCF-7 breast cancer and ECC1 endometrial cancer cells measured by real-time qRT-PCR.** MCF-7 and ECC1 cells were treated with $E_2$ (1nM) for 2, 4, 8, 16, 24 or 48 h, and expression of XBP1 was measured using real-time qRT-PCR and compared with vehicle treated cells (**A & C**). MCF-7 and ECC1 cells were treated with Cycloheximide (10μg/ml), 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (75μM), or fulvestrant (1mM) in absence or presence of $E_2$ (1nM) for 24 hrs and expression of XBP1 was assessed using real-time qRT-PCR (**B & D**).

**Effect of XBP1 Depletion on Estrogen-Induced growth of MCF-7 and ECC1 Cells**

We used a pool of four short interfering (si) RNA to knock-down the XBP1 expression in MCF-7 and ECC1 cells, and investigated its effect on the estrogen-induced growth. The siRNA was able to deplete the XBP1 transcript levels significantly as confirmed by quantitative real-time PCR (**Figure 21A, 21C**). The cells transfected with XBP1 siRNA or control siRNA were re-seeded in 24 well plate and growth was of the cells were monitored over six day period in the presence or absence of 1nM estrogen. The estrogen mediated growth of MCF-7 and ECC1 cells were inhibited by 49 and 30 percent respectively, in the XBP1 depleted cells as compared to growth of control siRNA treated cells (**Figure 21B, 21D**). The basal growth (vehicle treated) of cells was also modestly inhibited in MCF-7 cells. The data clearly suggested that XBP1 plays a critical role in mediating the estrogen induced growth in ER positive breast and endometrial cancer cells.
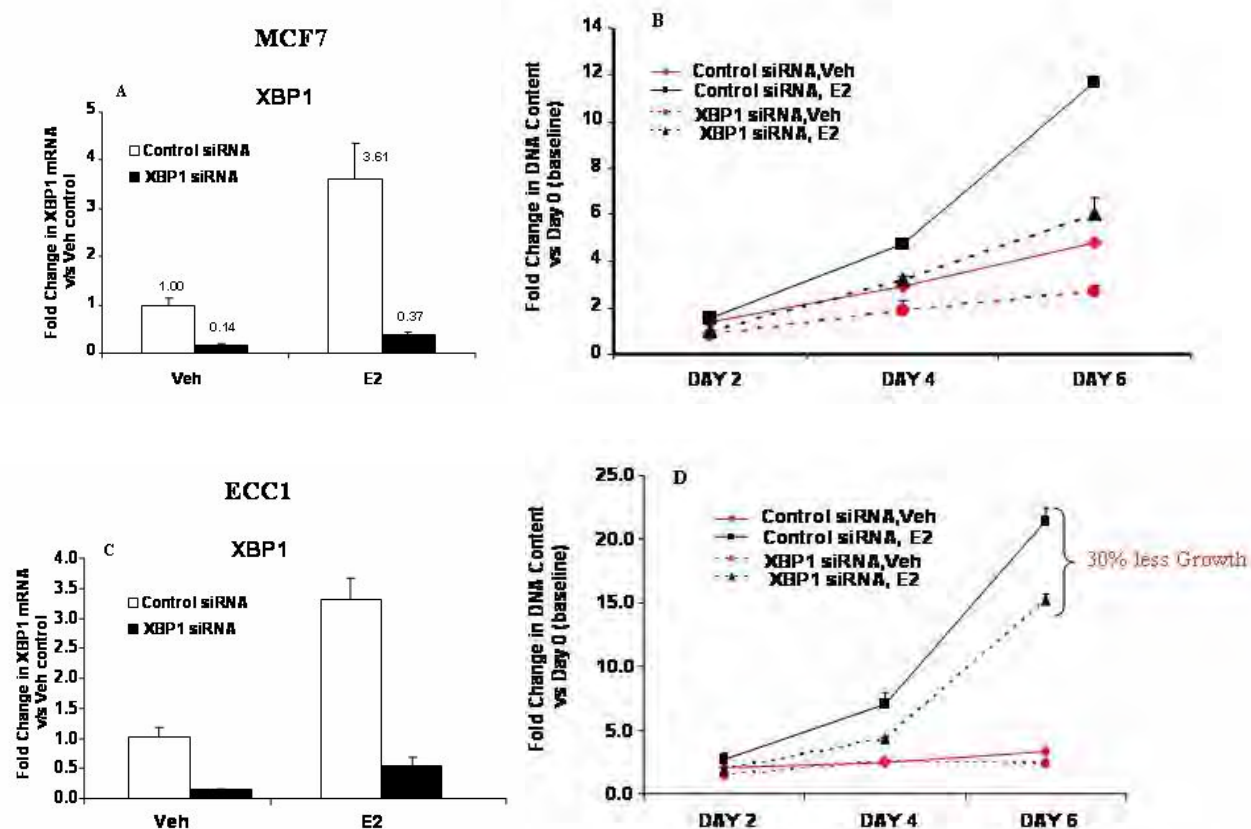
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**Figure 21. XBP1 depletion inhibits growth of MCF-7 and ECC1 cells.** MCF-7 and ECC1 cells, transfected with XBP1 siRNA or control siRNA, were treated with $E_2$ (1nM) or vehicle for 24 hrs and the extent of knock-down was assessed using quantitative real time PCR compared with control siRNA, vehicle treated cells (A & C). Subsequently cells were re-seeded and the growth of the cells was monitored over six day period. Total DNA content was measured as a marker of growth and the fold change in DNA content was calculated compared to the number of cells at the time of start of the treatment (baseline) (B & D).

## Effect of XBP1 depletion on expression of BCL2 (B Cell Lymphoma 2)

Since depletion of XBP1 severely impaired the estrogen mediated growth of cells we investigated the estrogen mediated regulation of several estrogen-responsive genes in XBP1 depleted cells. One of the gene whose expression was severely affected by the low levels of XBP1 is B cell lymphoma 2 (BCL2) gene. BCL2 is an oncogene, known to be up-regulated by estrogen in breast cancer cells and is also co-expressed with estrogen receptor alpha in breast cancer patients. Our study revealed that estrogen mediated up-regulation of BCL2 mRNA and protein was drastically suppressed in the XBP1 depleted MCF-7 (**Figure 22A, 22B**) and ECC1 (**Figure 22C, 22D**) cells. Interestingly, the basal expression level of BCL2 mRNA and protein was also inhibited in XBP1 depleted cells. This data suggested that XBP1 is a key regulator of the BCL2 gene expression in these cells.

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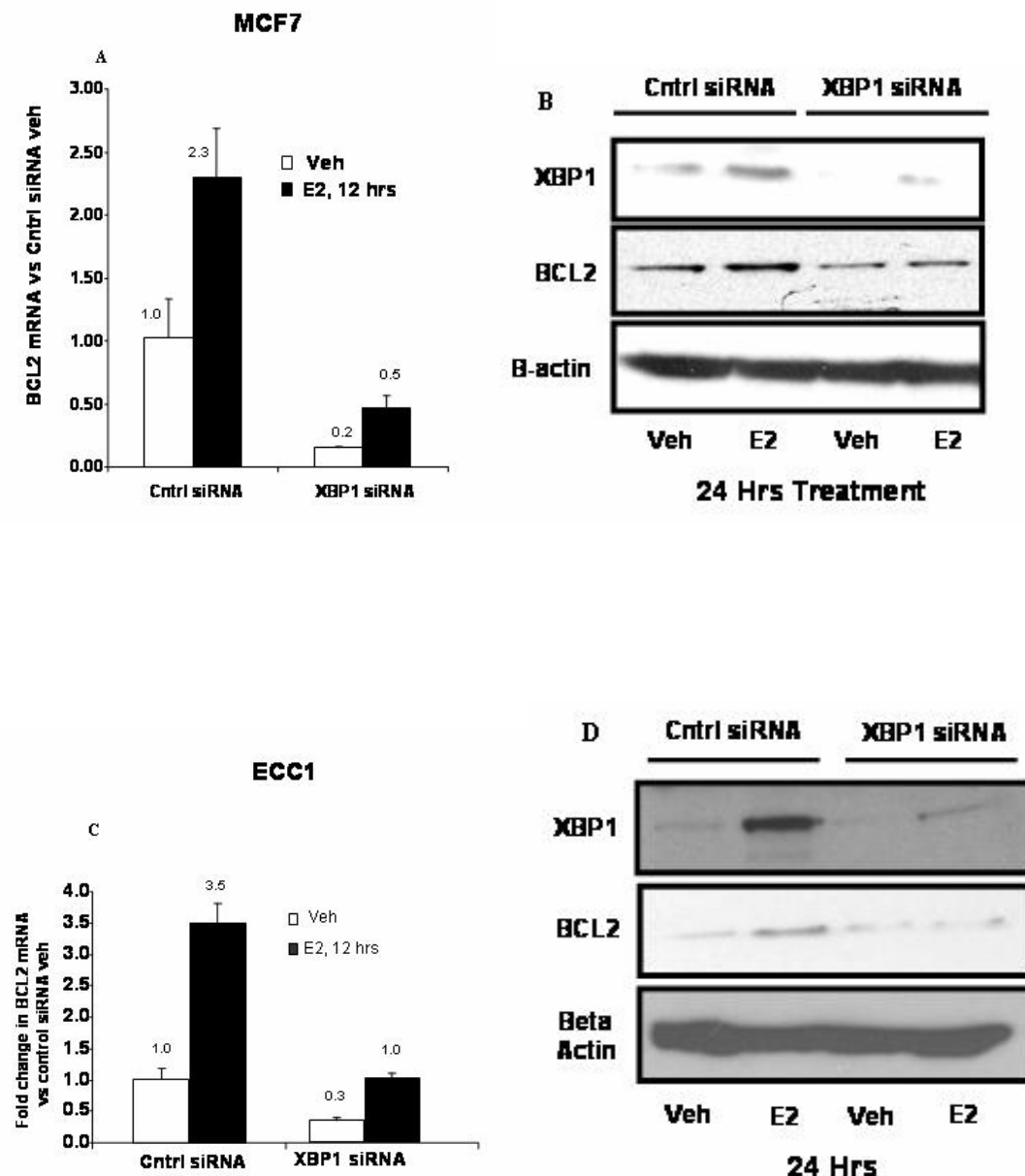
**Figure 22. XBP1 depletion inhibits BCL2 expression.** MCF-7 cells were transfected with XBP1 siRNA or control siRNA, and BCL2 mRNA expression was measured using real time q-RT-PCR (**A**) and BCL2 protein level were measured by western blotting (**B**) after 12 hr and 24 hr of estrogen treatment respectively. Similar results were observed using endometrial cancer, ECC1 cells (**C & D**).

## Recruitment of XBP1, Estrogen Receptor and other Factors at the Putative XBP1 Binding Site of BCL2 Promoter

XBP1 is a transcription factor which can bind to several DNA motifs with the core sequence of "ACGT" and regulate transcription. We therefore bio-informatically analyzed the promoter of BCL2 gene for putative XBP1 binding site. We first retrieved the sequence of approximately 3500 bases of BCL2 promoter spanning from around ~2500 bps upstream to ~1000 bps downstream of transcription start site (TSS). A putative XBP1 binding site, "GTGACGT" was located at 838 bp upstream of TSS (**Figure 23A**). We further arbitrarily divided the 3500 bps BCL2 promoter region into four equal regions. Each region was ~900 bps long and primers were designed for each region for quantitative real time PCR studies. The putative XBP1 binding site was located in the region area-two of the promoter (**Figure 23A**). Chromatin immunoprecipitation (ChIP) assay coupled with quantitative real time PCR was performed in MCF-7 cells to assess the recruitment of XBP1, ERα, nuclear corepressor (NCoR), phosphorylated RNA polymerase II and acetylated histone to the each region of BCL2

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promoter sequence. MCF-7 cells were treated with either vehicle or estrogen (1nM) for 45 minutes before isolating the fixed chromatin and performing ChIP assay. We found maximal recruitment of XBP1, ER alpha, and acetylated histone at the area two of BCL2 promoter (**Figure 23B, 23C, 23D**). Notably, the recruitment of these factors was independent of estrogen treatment. However, higher recruitment of phosphorylated RNA polymerase II was observed after estrogen treatment, indicating that estrogen induced the transcription of BCL2 (**Figure 23E**). De-recruitment of NCoR, a transcriptional corepressor, was observed after estrogen treatment (**Figure 23F**) suggesting its involvement in repressing the XBP1 transcription in absence of estrogen.
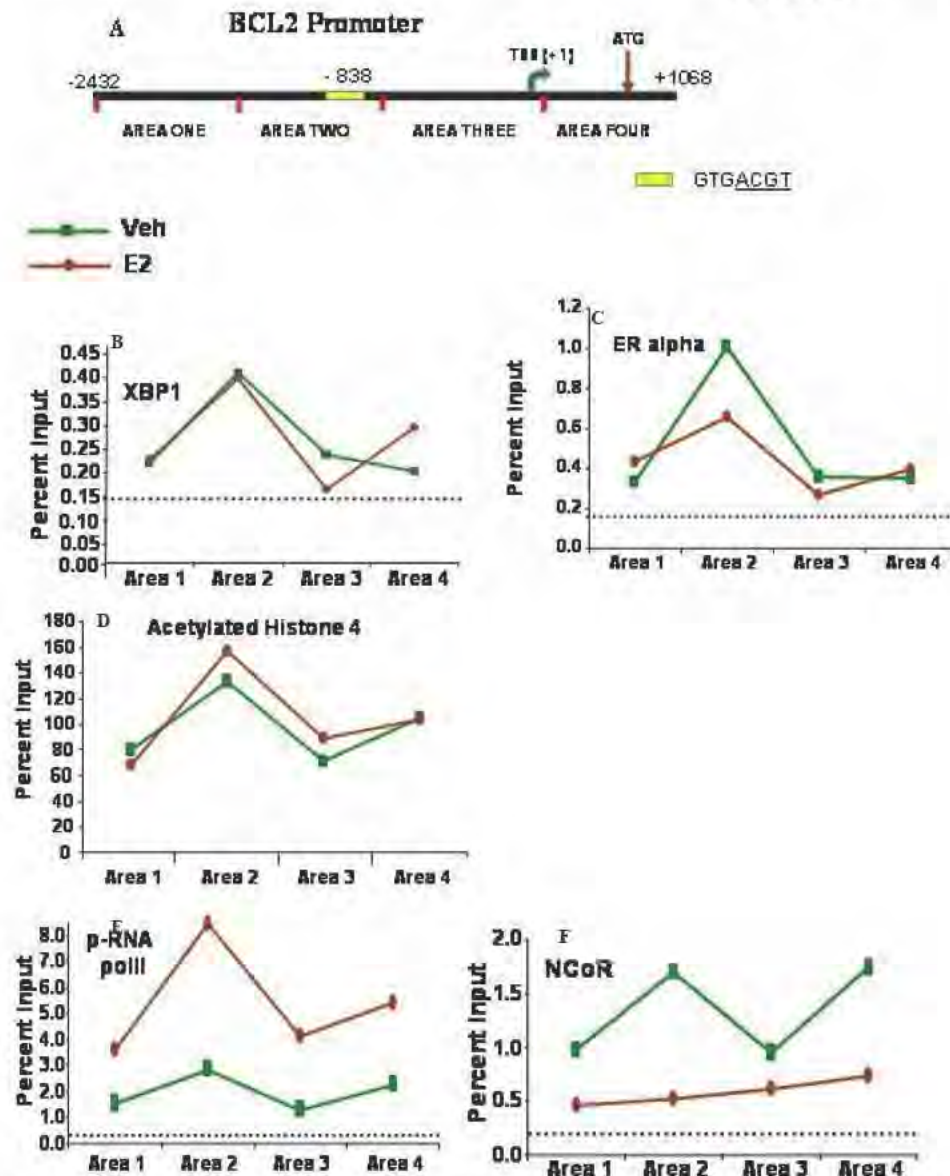


**Figure 23.** Schematic diagram of the BCL2 promoter region and the location of the putative XBP1 binding site along with the arbitrarily divided four regions labeled as area one to four (**A**). MCF-7 cells were treated with vehicle or 17-β estradiol for 45 minutes and fixed with 1% formaldehyde before isolating the soluble chromatin complex. Chromatin was sonicated, pre-cleared and subjected to immuno-precipitation using specific antibodies as mentioned in each panel (B to E). The extent DNA bound to specific proteins were assessed by quantitative real time PCR, using specific primers for individual BCL2 promoter regions, area one to four. The data is expressed as percent input of 5% of the starting chromatin used in each case. The dotted line in graph represents the maximum non-specific pull down using normal rabbit IgG.

In summary, this study illustrate that XBP1 is a critical player in the regulation of expression of proto-oncogene BCL2 in ERα positive breast and endometrial cancer cells and this novel mechanism for the regulation of

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cancer cell survival via XBP1 may be exploited as a novel drug target in future studies of anti-hormonal resistance in ER positive cancer cells. These studies are ongoing and will proceed during the 3rd year of the grant. **Figure 24** illustrates the elevation of both XBP1 and BCL2 in our estrogen deprived and resistant cells MCF-7:2A and MCF-7:5C cells compared to wild type MCF-7 cells.



**Figure 24.** Basal levels of XBP1 and BCL2 in estrogen-deprived resistant MCF-7:5C and MCF-7:2A cells as compared to parental MCF-7 cells measured by real-time qRT-PCR. Total RNA was isolated from these cells 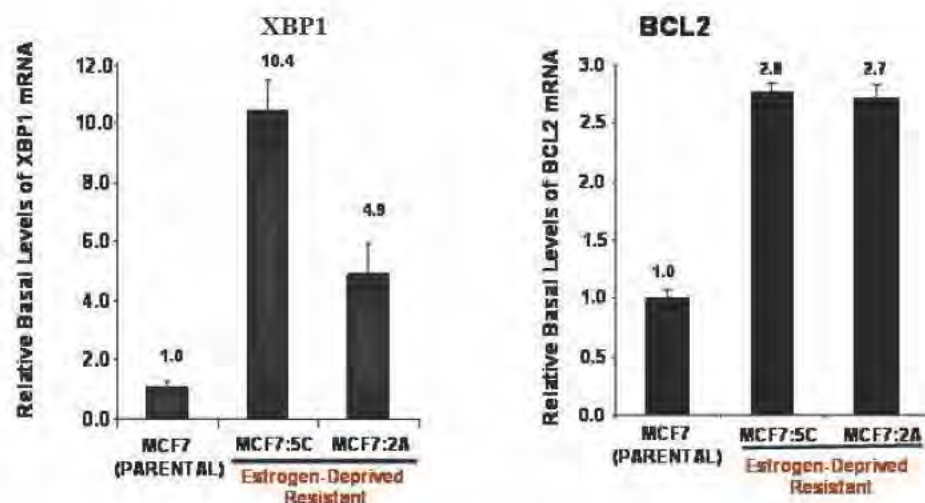and cDNA was synthesized using reverse transcriptase. Resulting cDNA was used to perform quantitative real-time PCR using specific primers for XBP1 and BCL2.

**TASK 3 – GEORGETOWN – Studies carried in the laboratories of Dr. Anna Riegel and Dr. Anton Wellstein**

**Task 3. To decipher cellular signaling pathways using proteomics and to mesh proteomics and mRNA analysis.**

Here we report work completed at the GU site during year 2 of this COE on proteomics and pathway analysis.

**PROTEOMICS AND PATHWAY ANALYSIS OF SURVIVAL AND APOPTOSIS**

The report is divided into two sections that reflect the contribution of different components (**Figure 20**):

1. **Proteomic MS analysis**. We report on $E_2$ effects in MCF-7 vs. MCF-7:5C vs. MCF-7:2A.

2. **Pathway analysis**. We report on data integration and pathway analysis.
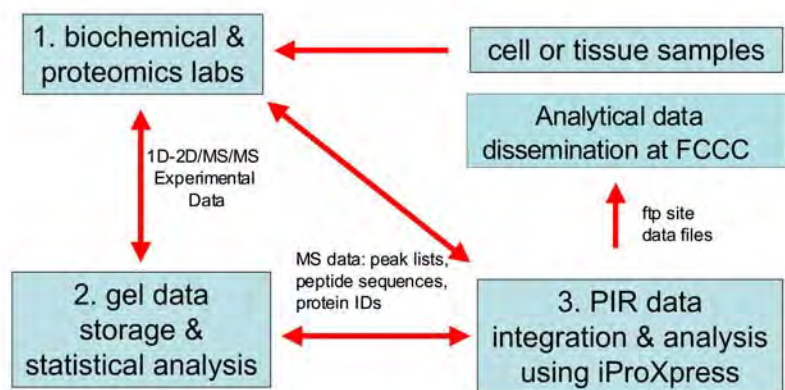


**Figure 25**. **Flow of samples and analytical data within the GU site (1, 2 and 3) and integration with the overall COE.** The three components of the GU site of the COE as well as the interface with FCCC are depicted. NOTE: This report is organized along the three components of the GU COE site.

**1. Proteomic MS analysis**
Mass spectrometry (MS) analysis of the proteome from $E_2$-treated MCF-7 (growth) versus MCF-7:5C cells (apoptosis) and versus MCF-7:2A (apoptosis) after fractionation by immunoprecipitation (IP).

**IPs.** From year 1, we continued proteome analyses of MCF-7:5C cells, which undergo apoptosis in response to $E_2$, and included MCF-7:2A cells which undergo delayed apoptosis relative to the MCF-7:5C cells, and compared these to wild-type MCF-7 that grow in response to $E_2$. The cells were cultured, and lysates prepared as described Under Task 2a (FCCC/Jordan, Ariazi). For the primary analysis, protein lysates were immunoprecipitated (IPed) using G-Sepharose beads and an AIB1 (or SRC-3) monoclonal antibody or an anti-phosphotyrosine (pY) monoclonal antibody (4G-10). The amount of input protein used for each set of IPs ranged between 7 mg and 14 mg.

**Gel electrophoresis.** The IPed proteins were resolved by denaturing SDS- PAGE on 4-12% Nu-PAGE gels (Invitrogen). After electrophoresis, gels were stained with colloidal blue overnight, and then washed with ddH$_2$0 overnight to reduce background staining.

**Isolation of distinctly regulated proteins**. Stained gels were imaged using a color scanner. These images were magnified and analyzed visually on a screen. Bands that were differentially represented were cut from the gels, as well as the same segment of all lanes from the different treatments. **Figures 26 and 27** show examples of proteins IPed using anti-AIB1 and anti-pY antibodies, respectively.
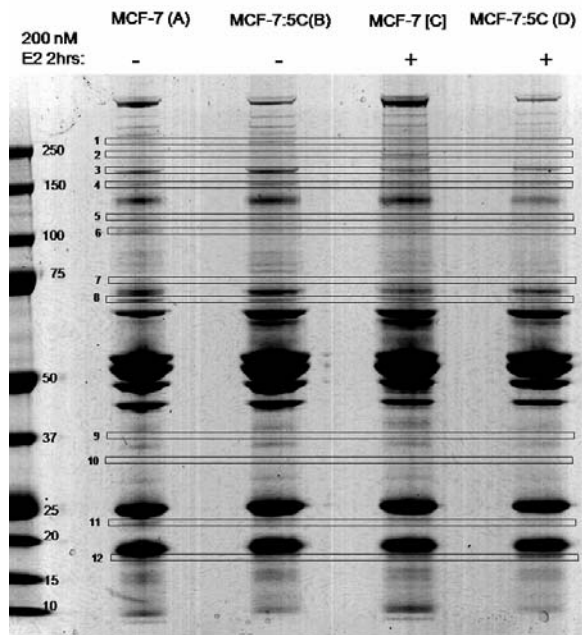


**Figure 26. Example of a colloidal-blue stained protein gel after IP of AIB1**. MCF-7 and MCF-7:5C cells were treated or not with E$_2$ for 2 hours, and then proteins were extracted and IPed with an anti-AIB1 antibody. Proteins separated by SDS-PAGE were stained and slices were cut from the gel for each segment that showed at least one distinctly regulated protein. The slices (1 – 12) and molecular weights of marker proteins are indicated (10 – 250 kDa). **High-resolution gel images will be posted at the COE website. Results from the experiments are discussed under section "2. Pathway analysis".**
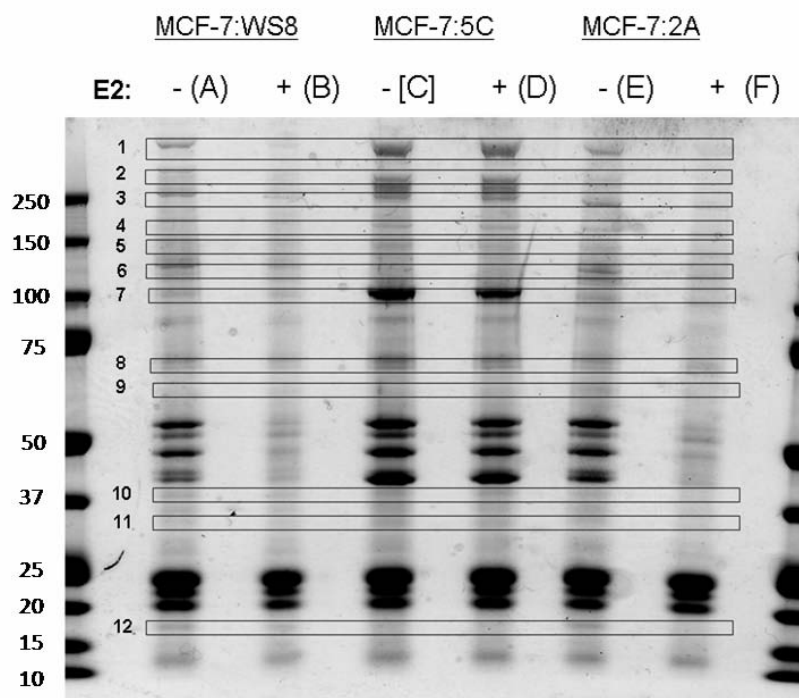
**Figure 27. Example of a colloidal-blue stained protein gels after IP of phospho-tyrosine (pY) proteins.** MCF-7 (left), MCF-7:5C (center) and MCF-7:2A (right) cells were treated or not with E$_2$ for 2 hours, and then proteins were extracted and IPed with an anti-pY antibody. SDS-PAGE and harvesting of gel slices was carried out as in **Figure 26**.

**Mass spectrometry analysis of isolated proteins.** Gel slices were subjected to tryptic digestion and analyzed using MS and tandem MS (MS/MS). Proteins corresponding to the peptide MS data were identified using the Mascot search engine database, which integrates MS readings and protein sequence analysis.

Overall, seven separate experiments have been run (4 repeats for IP anti-pY and 3 for anti-AIB1). Each experiment contained a head-to-head comparison of MCF-7:5C and MCF-7, or MCF-7:5C, MCF-7:2A, and MCF-7, without and with E$_2$ treatment. By the end of year 2, 410 gel slices will have been cut from the gels based on differential staining of proteins in the cross-comparisons. **Figure 26** (IP-AIB1) and **Figure 27** (IP-pY) provide some illustration from 2 of the experiments. The IP/1D-gel experiments were repeated three times each (IP-AIB1, and IP-pY), and independently analyzed by MS/MS. **Table 1** summarizes the experimental approach and numbers of gel slices harvested. Typically, each of the gel slices with a visible protein stain will contain as many as 10 distinct proteins that are detectable by MS sequencing. Some of the gel slices will contain less detectable proteins or less amounts of given proteins. These slices served as negative controls.

| Cell Lines Compared | IP | # of gel slices harvested |
|---|---|---|
| MCF-7, MCF-7:5C | pY | 24 (12/lane) |
| MCF-7, MCF-7:5C | pY | 40 (10/lane) |
| MCF-7, MCF-7:5C | AIB1 | 52 (13/lane) |
| MCF-7, MCF-7:5C | AIB1 | 48 (12/lane) |
| MCF-7, MCF-7:5C | pY | 120 (10/lane) |

| MCF-7, MCF-7:5C | AIB1 | 56 (14/lane) |
| MCF-7, MCF-7:5C, MCF-7:2A | pY | 72 (12/lane) |

**Table 1**. **Overview of cell line comparisons by proteomics**. The different IP approaches and number (#) of distinct bands cut for MS analysis is shown. Fig. 2 and 3 depict the approach (rectangular boxes indicate the area of gels cut for analysis).

**Data communication to the Protein Information Resource (PIR) for iProXpress (integrated Protein eXpression) analysis.** The protein lists from the MS analysis were provided to the PIR for pathway analysis using iProXpress, as described below under "2. Pathway analysis." In addition to the protein spreadsheets derived from the Mascot search, raw MS data of peptide masses were uploaded to the PIR site. From an analysis of the latter the Mascot search can be verified and peptide modifications due to posttranslational modifications revealed. This type of analysis requires the raw data and extensive exchange of information between the lab and PIR as indicated by the two-way arrows in the flow diagram of **Figure 25**.

**Conclusions**
- We have successfully generated proteomic data of MCF-7, MCF-7:5C and MCF-7:2A cells treated with or without $E_2$ which resuts in growth, fast apoptosis or delayed apoptosis, respectively. Signaling complexes were isolated by IP with anti-pY and anti-AIB1 antibodies.
- By the end of year 2, we have isolated differentially regulated proteins in approximately 410 gel slices. These are continuously being analyzed by mass spectrometry and protein sequencing.
- We found that by 24 hours after $E_2$ treatment, apoptosis was fully initiated as indicated by the proteomics. We thus expanded the analysis to include earlier time points (2 hours) to identify proteins which "trigger" $E_2$-induced apopoptosis in MCF-7:5C and MCF-7:2A cells compared to MCF-7 cells.
- We found distinct $E_2$-induced proteomic signatures delineated by anti-pY IP in MCF-7, MCF- 7:5C, and MCF-7:2A cell, and by anti-AIB1 IP in MCF-7 and MCF-7:5C cells.
- We found in our analysis of phosophorylated proteins by MS that AIB1 was itself tyrosine phosphorylated, and that this event was induced by $E_2$ and by growth factors in MCF-7 cells. Additional experimentation revealed that this tyrosine phosphorylation occurred at residue Y1357. Phosphorylated Y1357 was increased in HER2/neu mammary tumor epithelia and was required to modulate AIB1-mediated coactivation of ER$\alpha$, progesterone receptor B isoform (PR-B), NF-$\kappa$B and AP-1 dependent promoters. Further, we found the c-Abl tyrosine kinase directly phosphorylated AIB1 at Y1357, and this event modulated the association of AIB1 with c-Abl, ER$\alpha$, the transcriptional cofactor p300, and the methyltransferase CARM1. AIB1-dependent transcription and phenotypic changes, such as cell growth and focus formation, could be reversed by an Abl kinase inhibitor, imatinib. Thus, the phosphorylation state of Y1357 can function as a molecular on/off switch and facilitates the cross-talk between hormone, growth factor and intracellular kinase signaling pathways in cancer. **This work was accepted for publication in Molecular and Cellular Biology, August 2008, and is attached as a manuscript entitled "Tyrosine phosphorylation of the nuclear receptor coactivator AIB1/SRC-3 is enhanced by Abl kinase and is required for its activity in cancer cells" by Oh et al.** We are currently determining the role of tyrosine phosphorylation of AIB1 in the $E_2$-induced apoptotic response in MCF-7-5C cells.

**2. Pathway analysis**

The major task for the second year of the project at GU-PIR was to provide functional analysis and interpretation of the proteomics data generated from the lab and to propose for validation a candidate list of proteins that are potentially involved in $E_2$-induced apoptosis in MCF-7/5C cells. Meanwhile, the iProXpress expression analysis system is continually being improved by integrating additional functional pathway data as it arises into its underlying knowledgebase.

The experimental data were derived from IP of AIB1-interacting or tyrosine-phosphorylated (Tyr-phosphorylated) proteins from samples of $E_2$-treated breast cancer cells, followed by 1D-gel and MS/MS protein identification. The iProXpress bioinformatics analysis system was used to provide protein mapping, functional annotation of identified proteins, and pathway and network analysis of the data. AIB1 was shown to interact with an enriched group of proteins specifically in $E_2$-treated cells, which are involved in RNA metabolism and transcription, and with functions including transcriptional regulation, chromatin interaction and regulation, and mRNA splicing. Interestingly, several of those proteins are known to induce apoptosis, *e.g.* Sirt3 and TLE3. Several Tyr-phosphorylated proteins in $E_2$-treated MCF-7/5C cells, such as CDK1 and CIP29, also have been associated with apoptosis. Pathway mapping suggested that proteins in G-protein coupled receptor signaling (GPCR) pathway was involved in $E_2$-induced apoptosis. For example, $G\alpha(o)$ was Tyr-phosphorylated and Rap1GAP was pulled down with AIB1, and it has been shown that $G\alpha(o)$ can directly activate Rap1GAP, which in turn inhibits Ras/MAPK cell growth-promoting pathway and also induces apoptosis in some cancer cells. In brief, several AIB1-IPed (Sirt3, TLE3, and Rap1GAP) and pY-IPed proteins ($G\alpha(o)$, CDK1, and CIP29) were specifically identified in $E_2$-treated MCF-7/5C cells, which are all associated with apoptosis, including the GPCR activation pathway, thus are potential targets for validation.

**Bioinformatic analysis.**
We adopted the bioinformatics strategy depicted in **Figure 28B**, and used the iProXpress expression analysis system [Huang et al., 2007] for function and pathway analysis of the proteomics data derived from $E_2$-treated MCF-7:5C cells. The iProXpress system contains three major components: a data warehouse with information derived from over 90 databases, analytical tools for sequence analysis and functional annotation, and a graphical user interface for protein mapping, functional annotation, and function and pathway profiling. The system's unique features include its comprehensiveness of protein sequence coverage and annotation, high protein mapping rate of expression data, and its versatility of use on different types of 'omics' data, as described in [Chi et al., 2006; Hu et al., 2008a, 2008b].



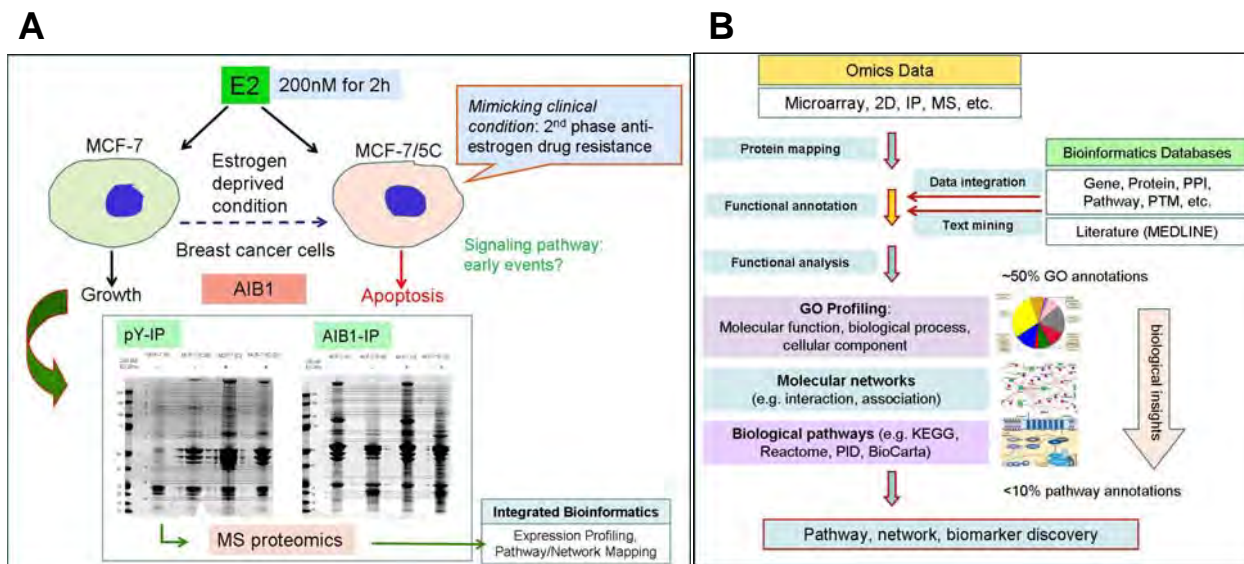**Figure 28**. **A.** Schematic drawing of experimental procedures. **B.** Strategy for bioinformatic analysis.

The MS proteomic data (protein identifications) were divided into a number of data groups based on source of samples (GU or FCCC) individual experiment (IP-AIB1/IP-pY, or repeats), lanes (+/- $E_2$), and single or tandem MS (scheme: *MS1/2"antibody type"[lab-source][cell type][time point]_"gel lane"*) **(Table 2)**. The data

grouping information was annotated for all identified proteins being integrated into the iProXpress system for direct data comparison between selected experimental groups.

Pathway and network analyses were assisted with Ingenuity Pathways Analysis (www.ingenuity.com) and GeneGO MetaCore (www.GeneGO.com) software tools.

| Data Group* | Sample Explanation | Experiment | | | |
|---|---|---|---|---|---|
| **Cell samples from GU** | | | MS1PYFC1W824_A MS1PYFC1W824_B | **Cell samples from FCC** FC1 – first experiment from FCCC W8 – MCF-7/WS8 cells (wild type) 24 – 24 hours | |
| MS1PY1_A MS1PY1_B ---------- MS2PY1_A MS2PY1_B | A – Lane A for MCF-7 cells (+) E2 B – Lane B for MCF-7/5C cells (+) E2 | First IP-pY using E2 treated MCF-7 and MCF-7/5C cells for 2h, by Ben. (date: March 2007) | MS2PYFC1W824_A MS2PYFC1W824_B MS1PYFC1W848_C MS1PYFC1W848_D ---------- MS2PYFC1W824_C MS2PYFC1W824_D | A – minus E2 B – plus E2 48 – 48 hours C – minus E2 D – plus E2 | The IP-pY samples from MCF-7:WS8 which are the wild-type cells FCCC uses, treated with or without E2 for 24, 48 and 72 hrs. |
| MS1PY40_A MS1PY40_B MS1PY40_C MS1PY40_D ---------- MS2PY40_A MS2PY40_B MS2PY40_C MS2PY40_D | A – Lane A for MCF-7 cells (-) E2 B – Lane B for MCF-7/5C cells (-) E2 C – Lane C for MCF-7 cells (+) E2 D – Lane D for MCF-7/5C cells (+) E2 | Second IP-pY using E2 treated MCF-7 and MCF-7/5C cells for 2h as well as untreated cells, by Ben. (date?) | MS1PYFC1W872_E MS1PYFC1W872_F ---------- MS2PYFC1W824_E MS2PYFC1W824_F | 72 – 72 hours E – minus E2 F – plus E2 | |
| MS1AIB1_A MS1AIB1_B MS1AIB1_C MS1AIB1_D ---------- MS2AIB1_A MS2AIB1_B MS2AIB1_C MS2AIB1_D | A – Lane A for MCF-7 cells (-) E2 B – Lane B for MCF-7/5C cells (-) E2 C – Lane C for MCF-7 cells (+) E2 D – Lane D for MCF-7/5C cells (+) E2 | First IP-AIB1 using E2 treated MCF-7 and MCF-7/5C cells for 2h as well as untreated cells, by Ben. (date?) | MS1PYFC15C24_G MS1PYFC15C24_H ---------- MS2PYFC15C24_G MS2PYFC15C24_H MS1PYFC15C48_I MS1PYFC15C48_J ---------- MS2PYFC15C48_I MS2PYFC15C48_J MS1PYFC15C72_K MS1PYFC15C72_L ---------- MS2PYFC15C72_K MS2PYFC15C72_L | 5C – MCF-7/5C cells 24 – 24 hours G – minus E2 H – plus E2 48 – 48 hours I – minus E2 J – plus E2 72 – 72 hours K – minus E2 L – plus E2 | The IP-pY samples from the MCF-7/5C cells treated with or without E2 for 24, 48 and 72 hrs |
| MS2AIB1R1_A MS2AIB1R1_B MS2AIB1R1_C MS2AIB1R1_D | R1 – Repeat 1 of the IP-AIB1 exp; A – Lane A for MCF-7 cells (-) E2 B – Lane B for MCF-7/5C cells (-) E2 C – Lane C for MCF-7 cells (+) E2 D – Lane D for MCF-7/5C cells (+) E2 | First repeat of IP-AIB1 using E2 treated MCF-7 and MCF-7/5C cells for 2h as well as untreated cells, by Ben. (date?) | MS1PYFC2WS8_A MS2PYFC2WS8_A MS1PYFC2WS8_B MS2PYFC2WS8_B | A – minus E2 B – plus E2 | IP-pY samples from FCCC for MCF-7/WS8 cells treated with or without 10-9 E2 for 2 hrs. (4/23/2008) |
| MS1AIB1R2_A MS1AIB1R2_B MS1AIB1R2_C MS1AIB1R2_D ---------- MS2AIB1R2_A MS2AIB1R2_B MS2AIB1R2_C MS2AIB1R2_D | R2 – Repeat 2 of the IP-AIB1 exp; A – Lane A for MCF-7 cells (-) E2 B – Lane B for MCF-7/5C cells (-) E2 C – Lane C for MCF-7 cells (+) E2 D – Lane D for MCF-7/5C cells (+) E2 | A second repeat of IP-AIB1 using E2 treated MCF-7 and MCF-7/5C cells for 2h as well as untreated cells, by Ben. (date?) | MS1PYFC25c_C MS2PYFC25c_C MS1PYFC25c_D MS2PYFC25c_D MS1PYFC22A_E MS2PYFC22A_E MS1PYFC22A_F MS2PYFC22A_F | C – minus E2 D – plus E2 E – minus E2 F – plus E2 | IP-pY samples from FCCC for MCF-7/5C cells treated with or without 10-9 E2 for 2 hrs. (4/23/2008) IP-pY samples from FCCC for MCF-7/2A cells treated with or without 10-9 E2 for 2 hrs. (4/23/2008) |

**Table 2**. Data groupings in iProXpress for the 1D-Gel/MS proteomic experiments.

## Results

Currently there are ~2200 proteins identified from proteomics experiments (Table 1) and integrated into the iProXpress system. They can be browsed and searched at http://pir.georgetown.edu/iproxpress/, (data file=coe2; password=coe234). Functional profiling and pathway mapping of AIB1-/pY-IPed proteins and comparison between different experimental conditions revealed some interesting groups of proteins and pathways potentially involved in E$_2$-induced apoptosis.

**RNA metabolism and transcription related proteins are major functional groups interacting with AIB1 in E$_2$ treated MCF-7/5C cells.**
Proteins specifically IPed with AIB1 in E$_2$-treated MCF-7/5C cells were profiled based on Gene Ontology (GO) (**Table 3**). In E$_2$ treated cells, GO *biological process* profiling shows that proteins in the category of transcription (9/32) and RNA metabolic process (11/32) are enriched in AIB1-IPed proteins compared to untreated cells. These proteins are listed in **Table 4**.

As shown in **Table 4**, these proteins are involved in transcriptional regulation, chromosome remodeling, chromatin interaction and histone regulation, as well as mRNA splicing and regulation. Several proteins are also known to be involved in apoptosis process, such as IASPP, TLE3 and Sirt3. Information regarding these gene processes was derived from the UniProt database or the literature.

| GO ID | GO Term | Frequency |
|---|---|---|
| GO:0000003 | reproduction | 1 |
| GO:0005975 | carbohydrate metabolic process | 2 |
| GO:0005976 | polysaccharide metabolic process | 1 |
| GO:0006066 | alcohol metabolic process | 2 |
| GO:0006118 | electron transport | 1 |
| GO:0006323 | DNA packaging | 1 |
| GO:0006350 | transcription | 9 |
| GO:0006396 | RNA processing | 1 |
| GO:0006457 | protein folding | 1 |
| GO:0006464 | protein modification process | 2 |
| GO:0006508 | proteolysis | 2 |
| GO:0006725 | aromatic compound metabolic process | 1 |
| GO:0006807 | nitrogen compound metabolic process | 1 |
| GO:0006810 | transport | 2 |
| GO:0006928 | cell motility | 3 |
| GO:0006936 | muscle contraction | 1 |
| GO:0007154 | cell communication | 7 |
| GO:0007155 | cell adhesion | 2 |
| GO:0008152 | metabolic process | 3 |
| GO:0008283 | cell proliferation | 2 |
| GO:0009308 | amine metabolic process | 1 |
| GO:0009987 | cellular process | 3 |
| GO:0015031 | protein transport | 1 |
| GO:0015931 | nucleobase\, nucleoside\, nucleotide and nucleic acid transport | 1 |
| GO:0016043 | cellular component organization and biogenesis | 2 |
| GO:0016070 | RNA metabolic process | 11 |
| GO:0016192 | vesicle-mediated transport | 1 |
| GO:0016458 | gene silencing | 1 |
| GO:0019538 | protein metabolic process | 2 |
| GO:0022607 | cellular component assembly | 1 |
| GO:0032501 | multicellular organismal process | 3 |
| GO:0032502 | developmental process | 6 |
| GO:0032989 | cellular structure morphogenesis | 1 |
| GO:0043062 | extracellular structure organization and biogenesis | 1 |
| GO:0043285 | biopolymer catabolic process | 3 |
| GO:0044237 | cellular metabolic process | 1 |
| GO:0050877 | neurological process | 3 |
| GO:0050896 | response to stimulus | 3 |
| GO:0051261 | protein depolymerization | 1 |
| GO:0051276 | chromosome organization and biogenesis | 1 |
| GO:0051641 | cellular localization | 2 |
| GO:0051704 | multi-organism process | 1 |
| GO:0065003 | macromolecular complex assembly | 2 |
| GO:0065007 | biological regulation | 14 |

| GO ID | GO Term | Frequency |
|---|---|---|
| GO:0000003 | reproduction | 1 |
| GO:0006082 | organic acid metabolic process | 1 |
| GO:0006118 | electron transport | 1 |
| GO:0006323 | DNA packaging | 1 |
| GO:0006350 | transcription | 1 |
| GO:0006412 | translation | 1 |
| GO:0006457 | protein folding | 1 |
| GO:0006464 | protein modification process | 3 |
| GO:0006508 | proteolysis | 1 |
| GO:0006519 | amino acid and derivative metabolic process | 1 |
| GO:0006629 | lipid metabolic process | 1 |
| GO:0006793 | phosphorus metabolic process | 1 |
| GO:0006810 | transport | 1 |
| GO:0006928 | cell motility | 1 |
| GO:0006996 | organelle organization and biogenesis | 1 |
| GO:0007049 | cell cycle | 1 |
| GO:0007154 | cell communication | 3 |
| GO:0008152 | metabolic process | 2 |
| GO:0008283 | cell proliferation | 1 |
| GO:0009308 | amine metabolic process | 1 |
| GO:0009987 | cellular process | 2 |
| GO:0015031 | protein transport | 1 |
| GO:0016032 | viral reproduction | 1 |
| GO:0016043 | cellular component organization and biogenesis | 3 |
| GO:0016070 | RNA metabolic process | 1 |
| GO:0016192 | vesicle-mediated transport | 2 |
| GO:0032501 | multicellular organismal process | 1 |
| GO:0032502 | developmental process | 3 |
| GO:0032989 | cellular structure morphogenesis | 1 |
| GO:0040011 | locomotion | 1 |
| GO:0044419 | interspecies interaction between organisms | 1 |
| GO:0050817 | coagulation | 1 |
| GO:0050878 | regulation of body fluids | 1 |
| GO:0050896 | response to stimulus | 2 |
| GO:0051261 | protein depolymerization | 1 |
| GO:0051276 | chromosome organization and biogenesis | 1 |
| GO:0051641 | cellular localization | 1 |
| GO:0065003 | macromolecular complex assembly | 1 |
| GO:0065007 | biological regulation | 6 |

**Table 3**. GO (biological process) profiles between $E_2$-treated (left) and untreated MCF-7/5C cells. RNA metabolism and transcription related proteins were found to be the major functional groups interacting with

| Functional Class | UniProtKB | Protein Name | Function and/or Pathway |
|---|---|---|---|
| Transcriptional regulation, chromatin interaction, histone regulation | Q8WUF5! IASPP_HUMAN | RelA-associated inhibitor (Inhibitor of ASPP protein) | NFkB inhibitor. Plays a central role in regulation of apoptosis and transcription via its interaction with NF-kappa-B and p53/TP53 proteins |
| | Q14765 STAT4_HUMAN | STAT4 | Jak-STAT signaling pathway (KEGG) |
| | P17027 ZNF23_HUMAN | Zinc finger protein 23 (Zinc finger protein 359) | ZNF23 **inhibits cell cycle progression** [PMID:17137575]; may be involved in transcriptional regulation |
| | P57071 PRD15_HUMAN | PR domain zinc finger protein 15 | Contains SET domain (e.g. in **histone N-methyltransferase**), may be involved in transcriptional regulation. |
| | Q9ULD4 BRPF3_HUMAN | Bromodomain and PHD finger-containing protein 3 | Contains bromodomain that is found in many **chromatin associated proteins**. Bromodomains can interact specifically with acetylated lysine. PHD fingers have been identified as binding modules of methylated histone H3 |
| | Q04726 TLE3_HUMAN | Transducin-like enhancer protein 3 (ESG3) | **Transcriptional corepressor** that binds to a number of transcription factors. The interacting protein CUL4B is required for histone H3 and histone H4 ubiquitination in response to ultraviolet and may be important for subsequent DNA repair |
| | O94906 PRP6_HUMAN | Pre-mRNA-processing factor 6 (PRP6 homolog) (PRPF6) | Part of **spliceosome**, involved in pre-mRNA splicing. *Reactome*: React_1675.1 (mRNA processing); PRP6-BRG1-NCoR [PMID: 12077342] |
| | Q9NTG7 SIRT3_HUMAN | NAD-dependent deacetylase sirtuin-3, mitochondrial precursor | Sirt3 is **pro-apoptotic** and participates in distinct basal apoptotic pathways [PMID:17957139] |
| mRNA splicing and regulation | Q9HAU5 RENT2_HUMAN | Regulator of nonsense transcripts 2 (Nonsense mRNA reducing factor 2) | Part of a **post-splicing** multiprotein complex involved in both mRNA nuclear export and surveillance. Involved in nonsense-mediated decay (NMD) of mRNAs containing premature stop codons. |
| | Q12926 ELAV2_HUMAN | ELAV-like protein 2 (Hu-antigen B) (HuB) | Binds to **3'-UTR** and regulate some transcription factor expression. |
| Ribonuclease | P12724 ECP_HUMAN | Eosinophil cationic protein precursor (ECP) (Ribonuclease 3) (RNase 3) | **Cytotoxin** and helminthotoxin with low-efficiency ribonuclease activity. Antibacterial activity. |

**Table 4**. AIB1-IPed proteins (11) enriched in RNA metabolism and transcription from $E_2$-treated MCF-7/5C cells.

**G-protein coupled receptor signaling pathway is involved in $E_2$-induced MCF-7/5C cell apoptosis.**
Pathway analyses of proteins specifically IPed from $E_2$-treated MCF-7/5C cellshave indicated several significantly represented pathways including GPCR signaling, apoptosis, integrin signaling, Huntington's disease signaling, and cytoskeleton remodeling. Of particular interest is GPCR signaling pathway **(Figure 29)**, in which Gα(o) was shown to be Tyr-phosphorylated and Rap1GAP associated with AIB1 specifically in $E_2$-treated MCF-7/5C cells, while Gα(o) can directly bind to Rap1GAP and modulate (inhibit) the RAS-MAPK cell proliferation pathway. Rap1GAP has emerged as an important cellular growth regulator and a putative tumor suppressor. It has been also shown to induce apoptosis when overexpressed in several tumor cells such as in pancreatic cancer cells [Zhang et al, 2006].

The integrin signaling pathway was also significantly enriched among proteins, *e.g.* FAK1, (IP-pY) and paxillin (IP-AIB1) from $E_2$-treated MCF-7/5C cells. Integrin pathway activation modulates cell mobility and can lead to gene regulation.
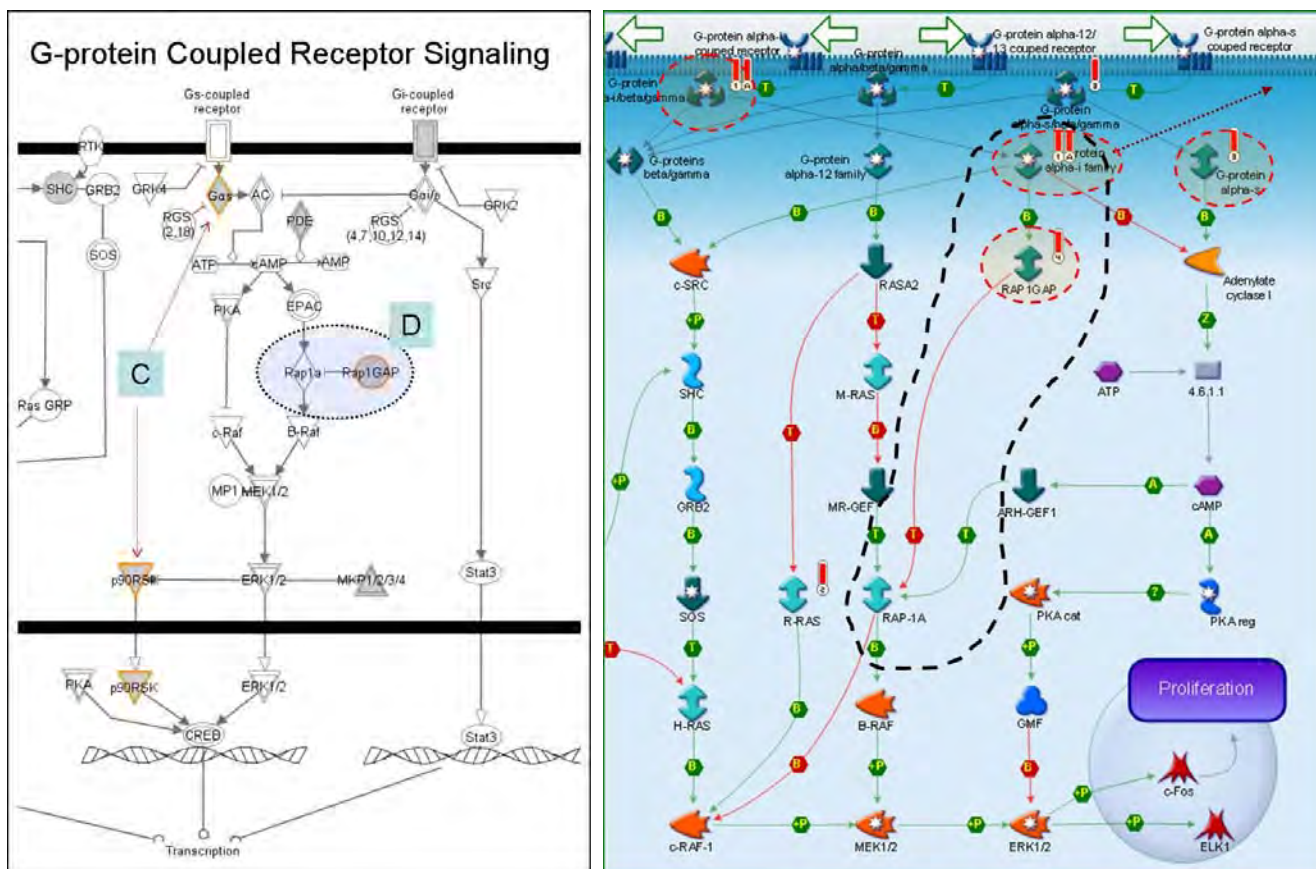
**Figure 29.** GPCR signaling pathway showing proteins IP-ed from $E_2$-treated MCF-7/5C cells. *Left*: Ingenuity pathway map. C – Tyr-phosphoryated-IPed proteins from $E_2$-treated MCF-7 cells; D – AIB1-IPed protein from $E_2$-treated MCF-7/5C cell. *Right*: MetaCore pathway map. Broken black enclosed area includes proteins of pY-IPed $G\alpha(o)$ and AIB1-IPed Rap1GAP from $E_2$-treated MCF-7/5C cells.

**Proteins in disparate pathways link to apoptosis.** Based on the above analysis, a number of proteins specifically identified in $E_2$-treated MCF-7/5C cells, either Tyr-phosphorylated or interacting/associating with AIB1, are proposed to be related or link to apoptosis. **Figure 30** depicts several of such proteins (green color for pY-IPed, red color for AIB1-IPed). Three pY-IPed ($G\alpha(o)$, CDK1 and CIP29) and three AIB1-IPed proteins (Rap1GAP, Sirt 3and TLE3) from $E_2$-treated MCF-7/5C cells are all linked to apoptosis, some of which interact with each other ($G\alpha(o)$-Rap1GAP, CDK1-Rap1GAP). Some of these proteins are cytoplasmic, and some nuclear, but all related to apoptosis, suggesting their dynamic movement within the cell in response to different signals.

Although there are many gaps in the pathways leading to apoptosis, it is feasible that $E_2$-induced apoptosis may involve the non-traditional GPCR pathway through membrane-associated ERα estrogen receptor, or through the G-protein coupled estrogen receptor GPR30. Although AIB1 is known to be a transcriptional coactivator, it is also known to shuttle between the nucleus and cytoplasm. It is possible that $E_2$ binds to membrane ERα in the cytosol and recruit AIB1, which in turn recruits/interacts with Rap1GAP and other cytoplasmic proteins. However, our proteomic experiments and pathway analyses did not reveal or provide information on what pathway may lead to Try-phosphorylation of these identified proteins, which require future investigation into these early events.
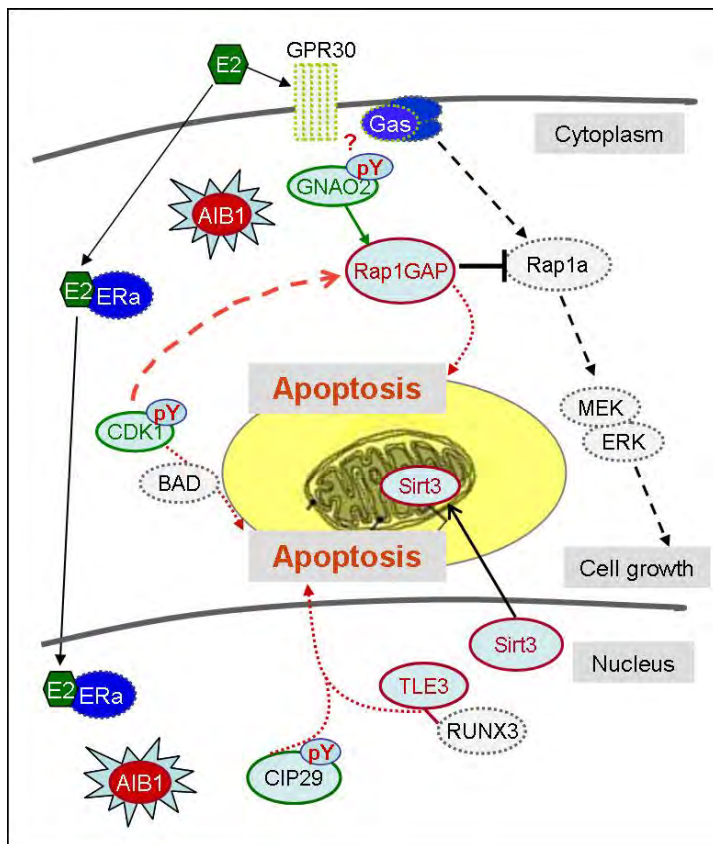
**Figure 30.** Pathways linking to apoptosis in E2 MCF-7/5C cells

## Conclusions and Future Directions

Functional and pathway analyses of the proteomics data using the iProXpress system have provided interesting and potentially important early signaling proteins involved in $E_2$-induced apoptosis in MCF-7:5C cancer cells.

We will continue to refine the protein enrichment and pathway analysis related to $E_2$-induced apoptosis in MCF-7/5C cells, including close examination and comparison of phosphotyrosine IPed proteins from cell samples separately prepared at GU and FCCC, and will then propose a focused group of proteins for experimental validation.

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**TASK 4 (FCCC/Jordan, Ariazi; in collaboration with TGen/Cunliffe): To analyze $E_2$-induced survival and apoptotic pathways using gene arrays and siRNAs**

**Task 4a. Catalogue the transcriptional response using array-based expression profiling.**

**Task 4b. Identify regulatory networks for pathways indicative of differential responses to $E_2$.**

**Overarching scheme of experiments in this task:** Array-based expression profiling of all *in vitro* and *in vivo* models generated under **Task 2** will be employed to identify genes and pathways associated with survival and apoptosis mechanisms.

Here we report work completed on Tasks 4a and 4b at the Fox Chase Cancer Center site during year 2 of this COE involving microarray analyses of the *in vivo* antihormone-resistant breast cancer tumor models.

**GENE EXPRESSION MICOARRAY ANALYSIS OF ANTIHORMONE-RESISTANT BREAST CANCER XENOGRAFT TUMOR MODELS**

**WORK ACCOMPLISHED**
The experiments involving generation of xenograft tumor samples for microarray analyses were reported in the Year 1 Progress Report for this award under Task 2a, and are described in detail in the publication by Ariazi et al., "Emerging principles for the development of resistance to antihormonal therapy: Implications for the clinical utility of fulvestrant" (J Steroid Biochem Mol Biol, 102: 128-138, 2006) (1). During year 1, RNA was isolated from these tumors and microarray chip hybridizations were conducted using both Affymetrix 54k Human Genome U133 Plus 2.0 and Agilent 22k Human 1A (V2) platforms.

During year 2 of this award, we analyzed the gene expression profiles of the *in vivo* antihormone-resistant compared to wild-type breast cancer tumor models.

1) One set of gene expression analyses were conducted to identify genes associated with the development of antihormone resistance by examining expression profiles of antihormone-resistant tumors versus wild-type tumors. The specific comparisons were:
   A. Phase I SERM-resistant MCF-7/RAL1 tumors treated with raloxifene versus wild-type MCF-7/E2 tumors treated with $E_2$ (**RAL1 tumors + RAL vs. WT tumors + $E_2$**).
   B. Phase II SERM-resistant MCF-7/RAL2 tumors treated with raloxifene versus wild-type MCF-7/E2 tumors treated with $E_2$ (**RAL2 tumors + RAL vs. WT tumors + $E_2$**).
   C. Phase II SERM-resistant MCF-7/TAM2 tumors treated with tamoxifen versus wild-type MCF-7/E2 tumors treated with $E_2$ (**TAM2 tumors + TAM vs. WT tumors + $E_2$**).
   D. Phase II aromatase inhibitor-resistant MCF-7/5C tumors treated with no $E_2$ (estrogen deprivation) versus wild-type MCF-7/E2 tumors treated with $E_2$ (**5C tumors - $E_2$ vs. WT tumors + $E_2$**).

2) A second set of gene expression analyses were conducted to identify $E_2$-regulated genes associated with tumor regression and apoptosis by examining the differences in expression profiles of aromatase inhibitor-resistant MCF-7/5C tumors versus wild-type tumors where both tumor types were treated with and without $E_2$. The specific comparisons were:
   A. Wild-type MCF-7/E2 tumors treated with $E_2$ to promote growth compared to the same tumor type treated with no $E_2$ (or $E_2$ withdrawn) (**WT tumors + $E_2$ vs. WT tumors - $E_2$**).
   B. Phase II aromatase inhibitor-resistant MCF-7/5C tumors treated with $E_2$ to induce regression and apoptosis compared to the same tumor type treated with no $E_2$ (or estrogen deprivation) (**5C tumors + $E_2$ vs. 5C tumors - $E_2$**).

**Summary of Experimental Design**

We have developed multiple xenograft breast cancer models of antihormone resistance to the selective estrogen receptor modulators (SERMs) tamoxifen (TAM) and raloxifene (RAL), and to estrogen deprivation as a surrogate for aromatase inhibitors (AIs) (1-10). These *in vivo* models of antihormone resistance were developed by either 1) serially transplanting wild-type estrogen-stimulated MCF-7 (MCF-7/E2) xenograft tumors into SERM-treated immune-compromised mice for several years to mimic treatment in the clinic, resulting in RAL-resistant (MCF-7/RAL1) and TAM-resistant TAM (MCF-7/TAM2) xenograft tumor models, or 2) culturing MCF-7 cells in the presence of a SERM or under estrogen-deprived conditions for several years *in vitro*, and then injecting the resistant cells into immune-compromised mice to generate RAL-resistant (MCF-7/RAL2) and AI-resistant (MCF-7/5C) xenograft tumors.

Using these models, we have defined Phase I and Phase II antihormonal resistance based on their growth responsiveness to $E_2$ (1-10). In prior studies, we have shown that Phase I SERM-resistant [*i.e.* MCF-7/RAL1 (1, 5)] tumors are growth stimulated in response to either SERMs or $E_2$ (1, 3-5), whereas Phase II SERM [MCF-7/RAL2 (1, 6) and MCF-7/TAM2 (1, 8, 9)] and Phase II AI-resistant [(MCF-7/5C (1, 10)] tumors paradoxically undergo $E_2$-induced regression due to apoptosis. We compared gene expression profiles across these antihormone-resistant breast cancer models to identify unifying and selective pathways involved in their etiology, and to identify genes involved in this newly discovered mode of apoptotic action of $E_2$. Gene expression profiling was conducted using both Affymetrics 54K Human U133 Plus 2.0 Arrays and Agilent 22K Human 1A (V2) Microarrays. Only genes exhibiting significant changes in expression that were cross-validated in both microarray platforms were considered. Differentially expressed genes were filtered for those genes that modulate estrogen receptor α (ERα) activities in antihormone-resistant tumors compared to wild-type MCF-7/E2 tumors, for those genes selectively associated with Phase II resistance, and for those genes differentially regulated by $E_2$ in the Phase II MCF-7/5C tumors and associated with tumor regression versus wild-type MCF-7/E2 tumors and associated with tumor growth.

**Methods**

**Xenograft MCF-7 Breast Cancer Tumors**

All xenograft tumor specimens were generated from experiments described in detail in a previous report (1) in which the growth properties of each *in vivo* xenograft tumor model were characterized. All procedures involving animals in the prior report had been approved by the Fox Chase Cancer Center's Internal Animal Care and Use Committee.

All animal studies had employed female ovariectomized athymic BALB/c nude (*nu/nu*) mice (Taconic, Hudson, NY, USA) at 5–6 weeks of age. In brief, tumor line models, namely MCF-7/E2, MCF-7/RAL1 and MCF-7/TAM tumors, were maintained by serial passage in animals (*in vivo*) by bilaterally transplanting 1 mm$^3$ tumor pieces (from other tumor bearing animals) into recipient mouse axillary mammary fat pads. This procedure was also used to generate tumors for study. The cell line models MCF-7/RAL2 and MCF-7/5C, were maintained in cell culture (*in vitro*), and bilaterally injected at 10$^7$ cells per site into the axillary mammary fat pads to generate tumors. In culture, MCF-7/RAL2 were maintained in phenol red-free MEM supplemented with 1 μM RAL plus 5% dextran-coated charcoal-treated bovine serum as previously described (6), and MCF-7/5C cells were maintained in phenol red-free RPMI-1640 plus 10% dextran-coated charcoal-treated fetal bovine serum as previously described (10).

$E_2$ was administered to mice using a subcutaneously implanted 0.3 cm $E_2$ silastic capsule. This subcutaneous 0.3 cm $E_2$ silastic capsule was shown to achieve a mean serum level of 83.8 pg/ml (308 pM) $E_2$ (11) and approximates perimenopausal $E_2$ levels in women. The FUL formulation corresponded to the clinical Faslodex preparation, which is a proprietary solution of FUL prepared in primarily ethanol and some castor oil as a slow

release-rate modifier. The clinical Faslodex preparation was administered as a 2 mg *sc* injection given 5 times per week, totaling 10 mg/week. RAL and TAM were administered to mice by gastric intubation at 1.5 mg/day 5 days per week.

## RNA Isolation

Frozen xenograft tumor material was homogenized by sonication using the Covaris S-2 tissue homogenization instrument (Covaris Inc., Woburn, Massachusetts, USA). Briefly, 100-400 mg of frozen tumor shavings were transferred to a pre-chilled borosilicate tube. 500ul of RLT Buffer (Qiagen RNeasy Mini kit product #74104, Valencia, CA) was added and the tumor sample and immediately sonicated in a 20 °C water bath in a Multitemp III thermostatic circulator (GE Healthcare Lifesciences/Amersham Biosciences, Pittsburg, PA) with one treatment cycle at 500 mV using the following parameters: 5 s at a 1% duty cycle (dc) and 100 cycles per burst (cb), then 30 s at 20% dc and 50 cb, then 30 s at 20% dc and 250 cb (software SonoLab v. 1.0.0, Covaris, Inc.). After sonication, 500 ul of TRIzol (Invitrogen, Carlsbad, CA) was added to the sample and total RNA was isolated according to the TRIzol protocol recommended by the manufacturer with the following additional purification: The aqueous phase obtained following the TRIzol organic extraction was combined with an equal volume of 80% ethanol, and the homogeneous mixture was transferred to an RNeasy Mini column (Qiagen). RNA was then extracted according to the manufacturer's recommendations. RNA integrity and purity was determined using a 2100 Bioanalyzer (Agilent Technologies; Palo Alto, CA) and a Nanodrop ND-1000 Spectrophotometer (Nanodrop; Wilmington, DE) prior to hybridization.

## Microarray Hybridizations

Gene expression profiling was conducted using both Affymetrix 54k Human Genome U133 Plus 2.0 Arrays (one channel direct hybridization format; Affymetrix, Santa Clara, CA), and Agilent 22k Human 1A (V2) Oligo Microarrays (two channel competitive hybridization format; Agilent Technologies, Santa Clara, CA).

For the Affymetrix Human Genome U133 Plus 2.0 arrays, biotin-labeled cRNA was prepared from 5 µg total RNA and hybridized to the chips according to manufacturer protocols. Preparation of biotin-labeled cRNA and Affymetrix array hybridizations were performed through Northwestern University's Feinberg School of Medicine Genomics Core Facility (Chicago, IL). The arrays were washed and stained using the GeneChip Operating Software (GCOS)/Microarray Suite fluidics script and protocol specified by the manufacturer. Affymetrix arrays were imaged with the GCOS controlled Scanner 3000. Affymetrix array hybridizations were quality controlled by evaluating scaling factors, average background levels, percent present calls, and 3'/5' ratios. In addition, graphical methods based on probe-level intensity distributions, implemented in the Bioconductor package *affyPLM* (NUSE and RLE plots; chip pseudo-images) were used to assess quality. Four replicate Affymetrix hybridizations per tumor group were conducted.

For the Agilent Human 1A (V2) microarrays, Cy3- and Cy5-labeled cRNA probes were prepared and hybridized to the chips according to the manufacturer's protocols. The competitive reference cRNA was pooled from 4 independent RNA samples, and 1.5 µg of the reference cRNA was competitively hybridized against approximately 3 µg of the test sample cRNA (scaled according to label incorporation efficiency). Agilent arrays were washed and then imaged with the Agilent model G2505B scanner. Agilent array hybridizations were quality controlled as previously described (12) by evaluating intra-array consistency using the 100 internal control oligodeoxynucleotide detectors printed 10 times each randomly across the chip. The largest median SD of the log2 ratios of these internal control detectors was 0.1297 with a minimum median intensity of at least 75 units. This SD is associated with a 99.9% confidence interval for the ratio fold change of 0.85 to 1.18. Graphical methods (*e.g.*, MA plots, boxplots of control probe intensities) were also used to assess inter-array variability. Two replicate Agilent hybridizations per tumor group were conducted for all comparisons, except for the MCF-7/5C plus/minus $E_2$ comparisons, in which 3 hybridizations were conducted.

**Data Processing**

It has been reported that approximately 37% of the probes in the Affymetrix U133 plus 2.0 array have errors in their original annotation due to cross-hybridization with splice variants or closely related genes, and more than 5,000 probe sets detect multiple transcripts (13). To ensure correct annotation of the Affymetrix U133 Plus 2.0 arrays, the perfect match probe sequences within probe sets were mapped to the whole genome (NCBI Build 36.3) and to RefSeq (Release 27) using the BLAST algorithm (14). Perfect match probe sequences within an Affymetrix probe set that mapped to single unique processed RNA transcripts with 100% identity (which necessitates that these mapped probes all had a corresponding Entrez identifier) were used to generate a modified chip definition file, while the remaining probe sets were eliminated from further analysis. Using this modified chip definition file, the Affymetrix probe set expression values were processed using the robust multi-array average (RMA) method (15) to perform background correction, between-array normalization, and summarization of probe-level intensities. Probe sets showing low expression intensity (<100) across all arrays were removed from further analysis. After filtering, 22,855 probesets representing 15,251 RefSeq RNA transcripts were tested for differential expression.

To also ensure updated annotation of the Agilent Human 1A (V2) arrays, probe sequences were filtered according to the Bioconductor annotation package for this platform (16). Agilent probes were removed if they (i) lacked a mapping to an Entrez gene identifier and; (ii) lacked any gene ontology (GO) mappings. Processed signal ratio values (ratios due to the 2 channel format) were obtained from Agilent Human 1A (V2) arrays using Agilent's Feature Extraction (FE) software (v9.1), which incorporates a spatial detrending background correction method, loess transformation for intensity-dependent within-array dye normalization, and a surrogate value substitution of very low intensity values near background. Agilent probes were removed if they, according to Agilent FE software, (i) showed poor spot quality measures on more than one array; or (ii) had intensities near background such that their expression values were replaced by surrogate values in the FE software across both channels and all arrays. After filtering, 16,411 probes representing 14,679 RefSeq RNA transcripts were used in differential expression analysis.

**Statistical Significance of Gene Expression Values**

Differential expression was assessed using empirical Bayes moderated one-sample (for Affymetrix data) or two-sample (Agilent data) t-statistics implemented in the Bioconductor package *limma* (17). For both microarray platforms, gene lists with corresponding expression values used for comparisons between groups were constructed in the R programming language. To cross compare between platforms, RefSeq transcript identifiers were used to associate Agilent probe identifiers with Affymetrix probe set identifiers. Only significant changes in gene expression that cross-validated on both microarray platforms at a P-value < 0.001 were considered for downstream analyses.

**Gene Enrichment and Pathway Analysis**

Gene enrichment and pathway analysis was conducted using MetaCore version 4.7 from GeneGo. GeneGo is a bioinformatics and pathway analysis set of applications that contains a manually curated database of published experimental data including kinase signaling pathways, transcriptional regulation pathways, and protein-protein, protein-DNA, protein-compound interactions. A p-value generated in MetaCore is based on a hypergeometric test of enrichment, and measures the probability of observing the number of selected genes (or more) mapping to a particular curated pathway (or network, process, etc.) by chance, as a function of the total number of selected genes, the number of genes in the pathway, and the size of the "full set" of all genes in all the curated pathways.

**Results**

**Production of wild-type MCF-7/E2 tumor samples for microarrays.**

MCF-7/E2 tumor cores were implanted into 15 ovariectomized athymic mice and separated into 3 groups of 5 mice each, or 10 tumors per group. The treatment groups were control (no treatment), 0.3 cm $E_2$ capsule *sc*, or

0.3 cm $E_2$ capsule *sc* plus the complete antiestrogen FUL. The MCF-7/E2 tumors grew robustly when treated with the 0.3 cm $E_2$ capsule, but did not grow in the control group (**Figure 31**, $E_2$ vs. Control, $P < 0.0001$), demonstrating that these tumors were dependent on $E_2$. The average cross-sectional area of the MCF-7/E2 tumors treated with $E_2$ plus FUL was significantly smaller than that of tumors treated with $E_2$ alone (**Figure 31**, $P$-values $<0.0001$). Therefore, FUL inhibited $E_2$-stimulated growth of MCF-7/E2 tumors. For microarray analysis, only established MCF-7/E2 tumors grown in the presence of $E_2$ were used. Two weeks before the tumors were collected, the 0.3 cm $E_2$ capsule was removed from 2 mice to generate 4 $E_2$-withdrawn (or no $E_2$) control tumors.



**Figure 31. Growth of MCF-7/E2 Tumors Used for Microarray Analysis.**

**Production of Phase I-resistant MCF-7/RAL1 tumor samples for microarrays.**

MCF-7/RAL1 tumor cores were implanted into 20 ovariectomized athymic mice and separated into 4 treatment groups of 5 mice each (10 tumors/group) corresponding 1.5 mg/day RAL *po*, 0.3 cm $E_2$ capsule *sc*, 2 mg/day FUL *sc*, and control (no treatment). The MCF-7/RAL1 tumors were significantly stimulated to grow by RAL treatment ($P < 0.0001$) and by $E_2$ treatment ($P < 0.0001$) compared to control treatment (**Figure 32**). However, a modest amount of growth was observed in the control-treated group, indicating that these tumors are not absolutely dependent upon an ER ligand with partial agonist activity. FUL did not significantly effect the growth of MCF-7/RAL1 tumors (**Figure 32**). Thus, either a SERM or $E_2$, but not FUL, supports the growth of these MCF-7/RAL1 xenografts and classified this model as Phase I SERM-resistant. For microarray analysis, only established MCF-7/RAL1 tumors grown in the presence of RAL were used.
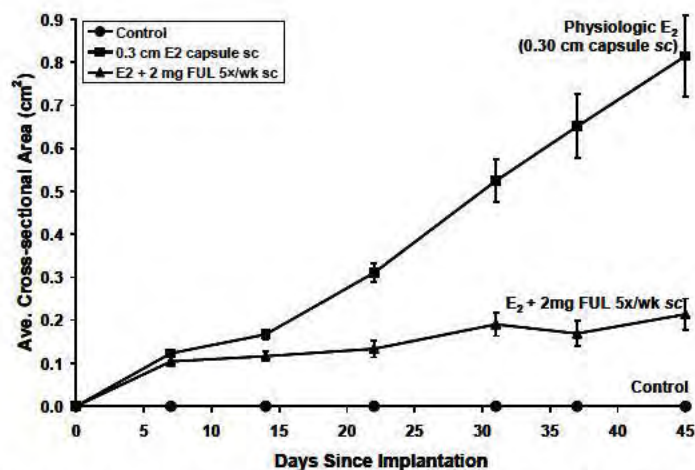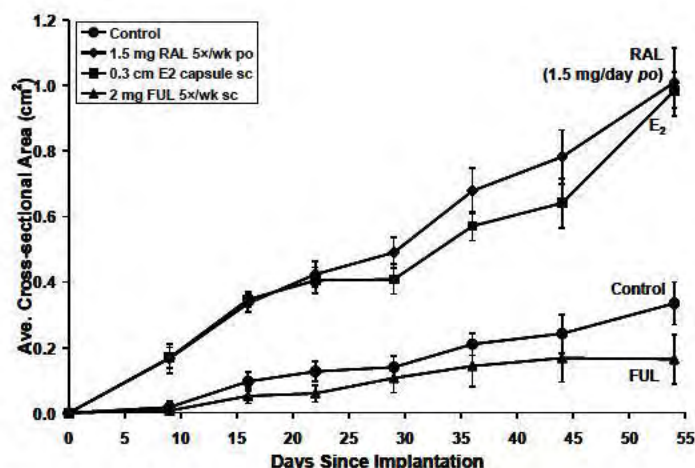


**Figure 32. Growth of MCF-7/RAL1 Tumors Used for Microarray Analysis.**

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**Production of phase II-resistant MCF-7/RAL2 tumor samples for microarrays.**

The MCF-7/RAL2 cells, propagated in culture, were injected at $10^7$ cells per site into 20 ovariectomized athymic mice, which were separated into 4 groups of 5 (10 tumors/group) and treated with 1.5 mg/day RAL *po*, 0.3 cm $E_2$ capsule *sc*, 2 mg/day FUL *sc*, or control (not treated). The MCF-7/RAL2 tumors only grew when treated with RAL (RAL vs. control, $P<0.0001$), and did not form any palpable tumors when treated with $E_2$, FUL or not treated (control) **(Figure 33)**. Therefore, growth of the MCF-7/RAL2 tumors was dependent on RAL, but inhibited by $E_2$ and FUL, which categorized these tumors as Phase II SERM-resistant. For microarray analysis, only established MCF-7/RAL2 tumors grown in the presence of RAL were used.
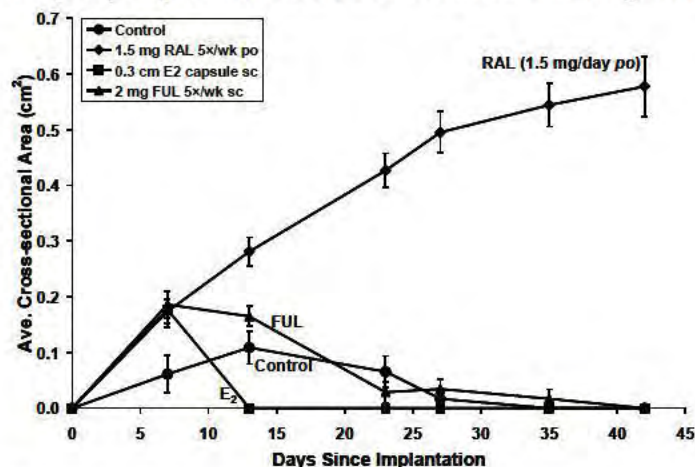


**Figure 33. Growth of MCF-7/RAL2 Tumors Used for Microarray Analysis.**

**Production of Phase II-resistant MCF-7/TAM2 tumor samples for microarrays.**

MCF-7/TAM2 tumor cores were implanted into 20 ovariectomized athymic mice, which were separated into 4 groups of 5 (10 tumors/group) and treated with 1.5 mg/day TAM *po*, 0.3 cm $E_2$ capsule *sc*, 2 mg/day FUL *sc*, or not treated (control). MCF-7/TAM2 tumors were stimulated to grow by TAM compared to the control group **(Figure 34**, $P < 0.0001$). FUL did not significantly effect growth of the MCF-7/TAM2 tumors versus control treatment. Interestingly, $E_2$ significantly inhibited tumor growth compared to the control group **(Figure 34**, $P = 0.0004$). Therefore, TAM stimulated growth, FUL did not support growth, and $E_2$ inhibited growth of MCF-7/TAM2 tumors, defining this model as Phase II SERM-resistant. For microarray analysis, only established MCF-7/TAM2 tumors grown in the presence of TAM were used.
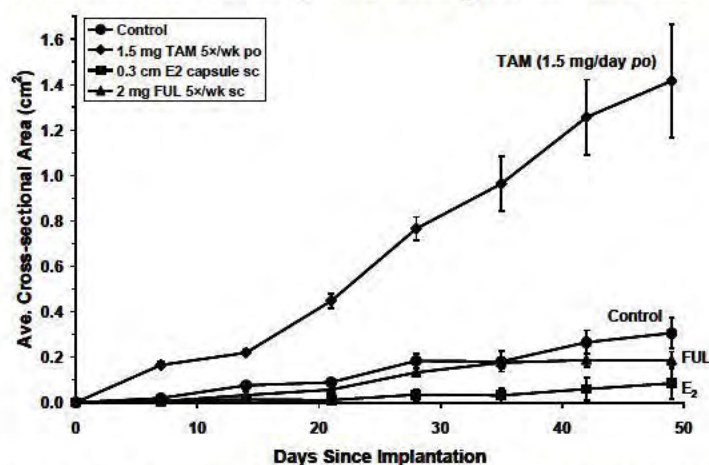


**Figure 34. Growth of MCF-7/TAM2 Tumors Used for Microarray Analysis.**

**Production of Phase II aromatase inhibitor-resistant MCF-7/5C tumor samples for microarrays.**

MCF-7/5C cells, propagated in culture, were injected into 20 ovariectomized athymic mice. The animals were separated into 4 treatment groups of 5 mice each (10 tumors/group), corresponding to control (not treated), 0.3 cm $E_2$ capsule *sc*, 2 mg/day FUL *sc*, and 0.3 cm $E_2$ capsule *sc* plus 2 mg/day FUL *sc*. MCF-7/5C cells rapidly

formed substantial tumors at every injection site (10 out of 10) in control-treated mice by 21 days after inoculation, but only 1 palpable tumor formed out of 10 injection sites in mice treated with $E_2$, resulting in a highly significant difference in the average tumor cross-sectional area between the two treatment groups (**Figure 35**, $P < 0.0001$), and defines this model as Phase II AI-resistant. Importantly, MCF-7/5C xenograft tumors showed robust growth in the presence of FUL or $E_2$ plus FUL, which was not significantly different than growth of the control (no treatment) group, but was significantly greater than in the $E_2$ treatment group (**Figure 35**, FUL vs. $E_2$, $P < 0.0001$; $E_2$+FUL vs. $E_2$, $P < 0.0001$). Hence, the MCF-7/5C xenograft tumor model was resistant to growth inhibition by FUL, and FUL treatment abrogated $E_2$-mediated growth inhibition. For microarray analysis, only established MCF-7/5C tumors grown in the absence of $E_2$ were used. However, two days before the tumors were collected, 3 mice were implanted with 0.3 cm $E_2$ capsules *sc* to generate 6 $E_2$-treated tumors.
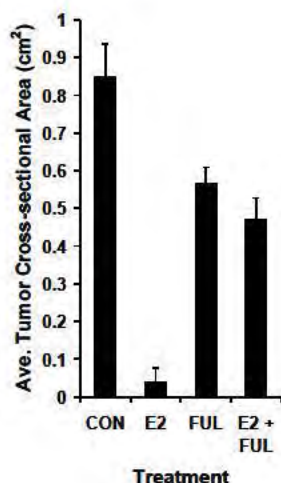


**Figure 35. Growth of MCF-7/5C Tumors Used for Microarray Analysis.**

**Comparisons of Gene Expression Profiles**

To identify genes associated with antihormone resistance, each of the antihormone-resistant tumor models were compared against the wild-type MCF-7/E2 tumors. Importantly, all tumor samples used had been grown under maximal growth conditions for the particular model in question, *i.e.* wild-type MCF-7/E2 tumors treated with $E_2$ (WT + $E_2$), MCF-7/RAL1 tumors treated with raloxifene (RAL1 + RAL), MCF-7/RAL2 tumors treated with raloxifene (RAL2 + RAL), MCF-7/TAM2 tumors treated with tamoxifen (TAM2 + TAM), MCF-75C tumors without $E_2$ treatment (5C − $E_2$). Similarly, to identify $E_2$-regulated genes selectively associated with tumor growth versus tumor regression, the wild-type MCF-7/E2 tumors and the AI-resistant MCF-7/5C tumors were both treated with $E_2$ [(WT + $E_2$), (5C + $E_2$)] and without $E_2$ [(WT - $E_2$), (5C - $E_2$)]. The specific comparisons to identify genes associated with antihormone resistance and $E_2$-induced tumor regression are represented in **Figure 36**.
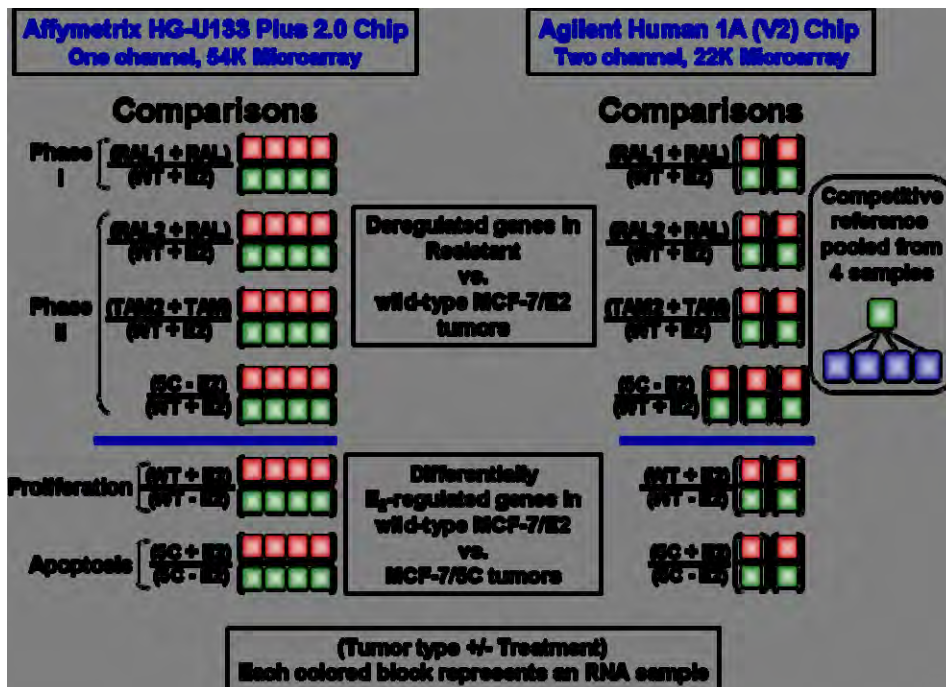
**Figure 36. Comparisons Used for Microarray Analysis.**

## Differentially Expressed Genes that modulate ER activities

Using Metacore GeneGo software, significantly differentially expressed genes were filtered for those that affect ER activities.

**Differentially expressed genes that modulate ligand-dependent activation of ERα (Figure 37)**

We observed that all the antihormone-resistant tumor models exhibited significantly decreased levels of the corepressor RIP140 (receptor interacting protein 140; NRIP1, nuclear receptor interacting protein 1). RIP140 interacts with ERα and c-Jun, and inhibits $E_2$-induced AP-1 mediated transcription (18). RIP140 also directly binds to histone deacetylases (e.g., histone deacetylase 4, HDAC4) and to the corepressor C-terminal binding protein 1 (CBP) which itself interacts with histone deacetylases (*e.g.*, histone deacetylase 1, HDAC1). Deacetylation of chromatin proteins by histone deacetylases leads to inhibition of ERα/ AP-1-induced transcription (18). As a negative transcriptional regulator of nuclear hormone receptors, decreased RIP140 levels would be predicted to derepress ligand-dependent activation of ERα, thereby allowing the development of antihormone resistance.

# Ligand-dependent Activation of ERα

17beta-Estradiol
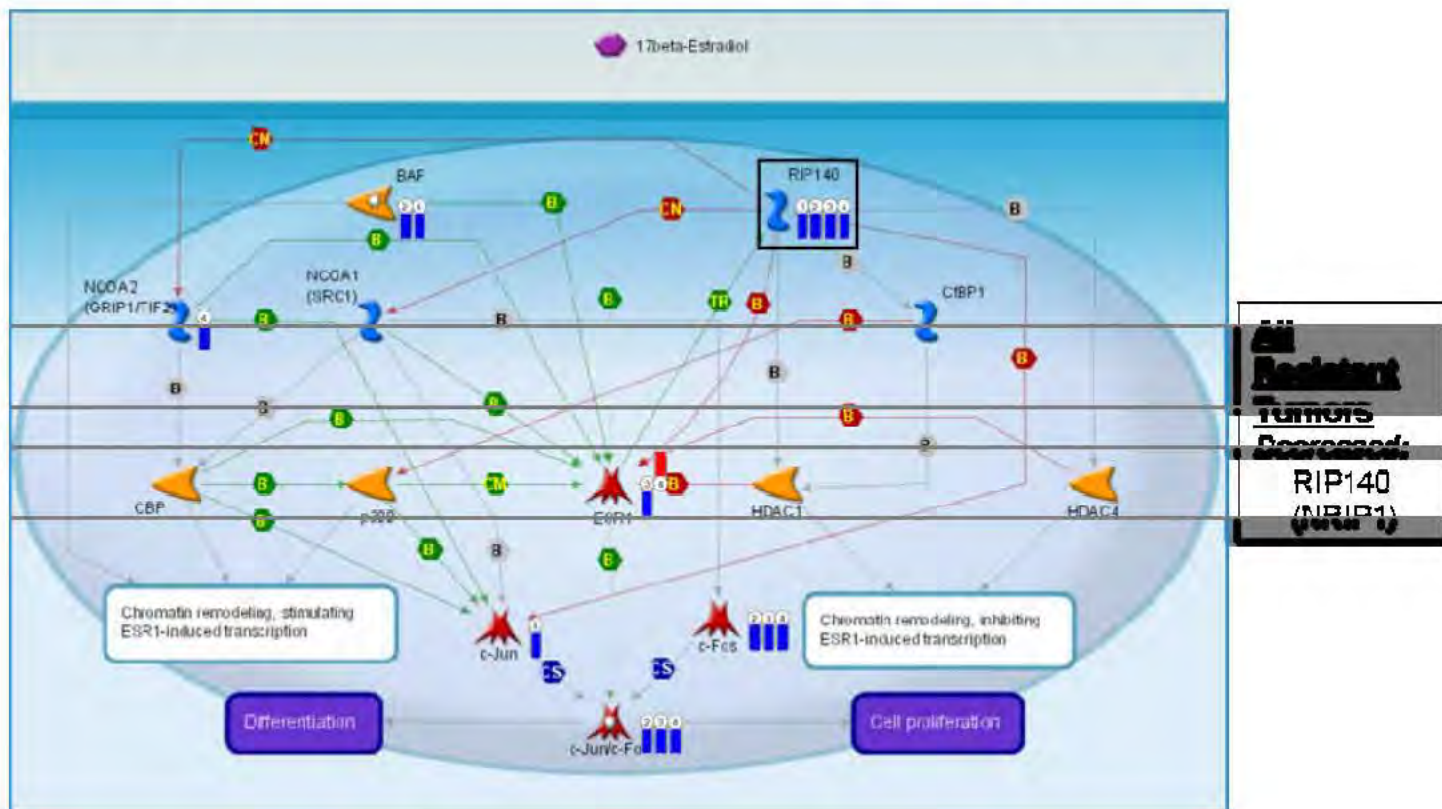
1. (RAL1 + RAL)/(WT + E2)    2. (RAL2 + RAL)/(WT + E2)
3. (TAM2 + TAM)/(WT + E2)    4. (5C - E2)/(WT + E2)

**Figure 37. Ligand-dependent activation of ERα canonical pathway as curated by GeneGo Metacore.** Red and blue bars indicate significantly increased and decreased expression, respectively. Numbers above the bars correspond to the specific comparison indicated under the diagram. Statistical significance was assessed at $P < 0.001$ and cross-validated in both the Affymetrix and Agilent microarray platforms.

## Differentially expressed genes that modulate non-genomic $E_2$ signaling through G-proteins (Figure 38)

In all the antihormone resistant tumors compared to wild-type MCF-7/E2 tumors, the G-protein guanine nucleotide-binding protein G(i) alpha-1 subunit (GNAI1) and phospholipase C beta-1 (PLCβ1) were decreased. Selectively in the Phase II antihormone-resistant compared to wild-type tumors, Caveolin-1 and c-Fos were decreased. ERα has been shown to localize to the plasma membrane through binding caveolin-1 (19) and striatin (20). $E_2$ can stimulate formation of a membrane ERα and G-protein α-i complex (20, 21), leading to MAPK and Akt activation (20), and through MAPK, lead to activation of the transcription factor Elk-1, which induces c-Fos expression (22, 23). Also, $E_2$-bound membrane ERα can activate PLCβ1 through G-protein alpha q. However, since GNAI1, PLCβ1, caveolin-1, and c-Fos were repressed, and these proteins can all mediate membrane ERα activities, it is likely that a role for membrane ERα was diminished in the development of these antihormone-resistant tumor models. As an aside from considering antihormone resistance, these results may also suggest that membrane ERα may have been important in the original development of maximal $E_2$-stimulated growth of the wild-type MCF-7/E2 tumors. Returning to consideration of antihormone resistance, the G-protein guanine nucleotide-binding protein subunit beta-5 (GNB5) was increased in all of the antihormone-resistant tumors compared to wild-type tumors. G-protein β subunit proteins can be activated by a novel estrogen-binding protein that is a G-protein coupled receptor termed GPR30 (24, 25). Interestingly, in addition to $E_2$, GPR30 also binds SERMs and fulvestrant as agonist ligands (26), and can mediate rapid non-genomic $E_2$

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signaling events (27). Hence, it is possible that GPR30 to GNB5 signaling substitutes for some membrane ERα activities in the development of antihormone resistance.
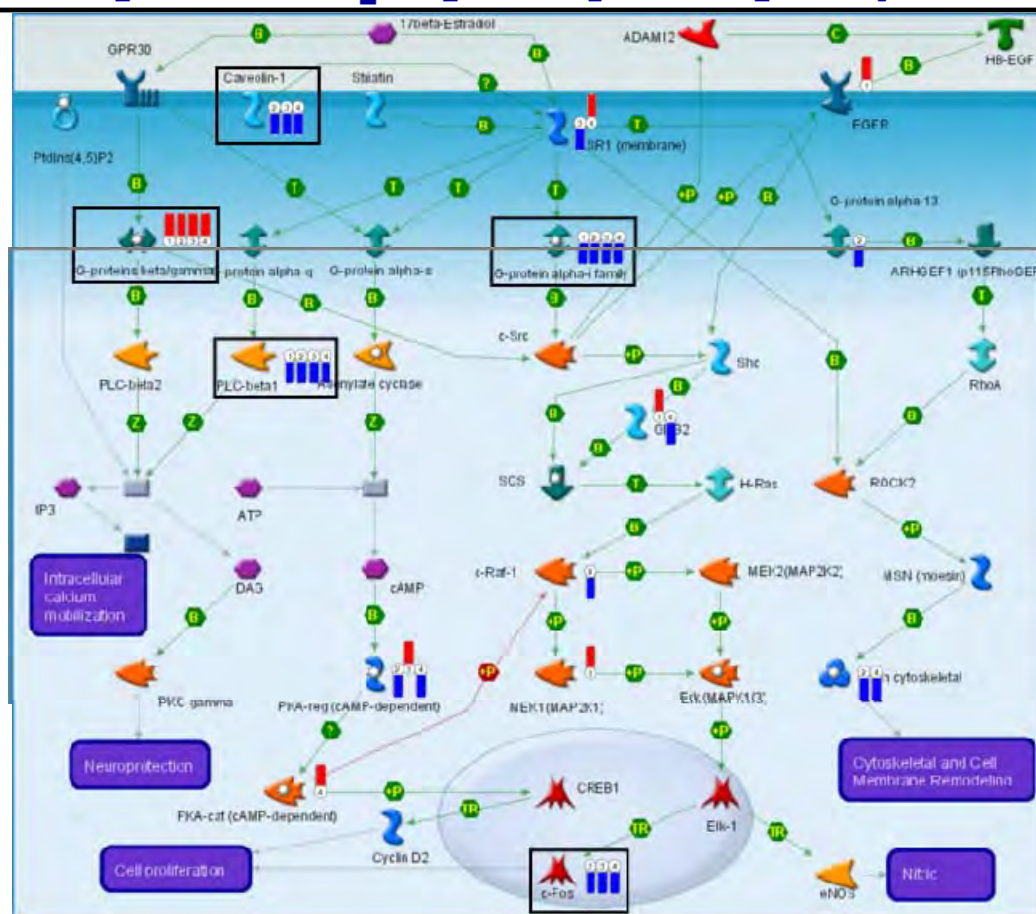


**Figure 38. Non-genomic E$_2$ signaling through G-proteins canonical pathway as curated by GeneGo Metacore.** Description of the figure icons is given in the legend to Figure 35.

**Differentially expressed genes that modulate ligand-independent activation of ERα (Figure 39)**
In all the antihormone-resistant tumors compared to wild-type MCF-7/E2 tumors, IGF-1 receptor was decreased. The membrane ERα can form a complex with (28, 29) and phosphorylate IGF-1 receptor (30), yet IGF-1 receptor was decreased in all the resistant models. This again indicates the lack of a role for membrane ERα in the development of antihormone resistance. However, pronounced ligand-independent ERα activity was indicated by selective alterations in signaling pathways depending on the particular resistant tumor model.

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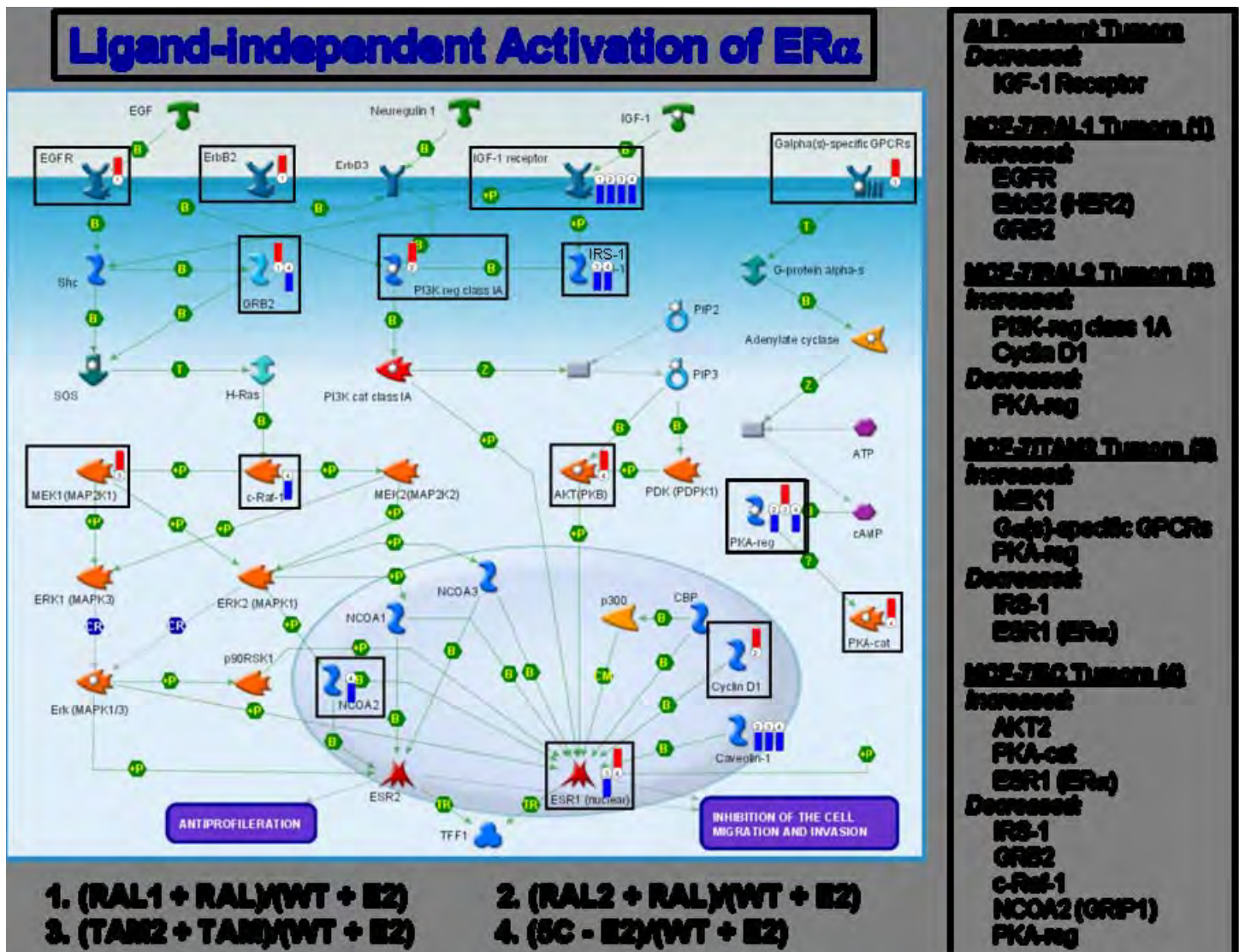
**Figure 39. Ligand-independent activation of ERα canonical pathway as curated by GeneGo Metacore.** Description of the figure icons is given in the legend to Figure 35.

In MCF-7/RAL1 tumors compared to wild-type MCF-7/E2 tumors, EGFR (epidermal growth factor receptor), ErbB2 (HER2) and Grb2 (growth factor receptor-bound protein 2) were increased. EGFR and HER2 each form homodimers and heterodimers, and these activated tyrosin kinase receptors then recruit Shc, GRB2, and SOS (son of sevenless) forming a protein complex. Activated SOS stimulates the small GTPase Harvey ras (H-RAS)**,** which stimulate the mitogen activated protein kinase (MAPK) cascade (31-33). This pathway ultimately targets ERα for phosphorylation at Ser118 and promotes its ligand-independent activation (22, 34-43). The MAPK cascade also phosphorylates the coactivators such as NCOA3 (nuclear receptor coactivator 3; AIB1, aimplified in breast cancer 1) (44-48). The EGFR and ErbB2 pathway has been shown to be etiologically involved in the development of antihormone resistance (5, 49-52). We have previously shown that MCF-7/RAL1 tumors overexpress EGFR and HER2 by real-time PCR, and that the clinically used ant-HER2 monoclonal antibody trastuzumab significantly inhibits growth of these tumors (5).

In MCF-7/RAL2 tumors compared to wild-type MCF-7/E2 tumors, PI3K-regulatory subunit class 1A [phosphatidylinositol 3-kinase regulatory subunit 1 (p85 alpha)] and cyclin D1 were increased, while PKA-regulatory subunits (cAMP-dependent protein kinase type I-alpha regulatory subunit and type II-beta regulatory subunit) were decreased. The PI3K to Akt pathway also plays an important role in the integration of receptor tyrosine kinase and ERα signaling by targeting phosphorylation of ERα-Ser167 (41, 46, 53-58). Cyclin D1 can act as a coactivator of ERα (59, 60) and hence potentiate its ligand-independent activity, however this ERα-

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cyclin D1 interaction is dependent on PKA (60). Perhaps a decrease in the PKA regulatory subunit would allow for a relative molar ratio increase in the PKA catalytic subunit, facilitating its activity and promoting ERα-cyclin D1 complex formation.

In MCF-7/TAM2 tumors compared to wild-type MCF-7/E2 tumors, MEK1 (mitogen-activated protein kinase kinase 1) Gα(s)-specific GPCR [D(1A) dopamine receptor] and PKA-regulatory subunit (type II-beta) were increased. In contrast, IRS-1 (insulin receptor substrate 1) and ERα levels were decreased. MEK1 is a critical component of the MAPK cascade and leads to Ser118 phosphorylation in ERα, and its unliganded activity as mentioned above (22, 34-43). Gα(s)-specific GPCR can lead to activation of the PKA regulatory subunit and hence the PKA catalytic subunit, which then leads to phosphorylation of ERα at Ser-305. ERα phosphorylation at Ser-305 regulates its dimerization (61), may block its acetylation at Lys-303 leading to enhanced transcription response (62), and increases its recruitment of NCOA3 (AIB3) (46). The decreased IRS1 in conjunction with decrease IGF-1 receptor indicated a de-emphasis of the IGF-1 receptor signaling to ERα.

In MCF-7/5C tumors compared to wild-type MCF-7/E2 tumors, ERα was increased. Also, Akt2 (RAC-beta serine/threonine-protein kinase), and PKA catalytic subunit (cAMP-dependent protein kinase, beta-catalytic subunit) were increased. However, Grb2, c-Raf-1, NCOA2 (nuclear receptor coactivator 2; GRIP1, glucocorticoid receptor interacting protein 1), IRS-1, and PKA-regulatory subunit (type I-alpha) were decreased. As discussed above, increased Akt2 expression can play an important role in promoting ligand-independent activation of ERα by phosphorylating it at Ser-167 (41, 46, 53-58). Additionally, Akt is intimately involved in promoting cell survival and antihormone resistance (54, 58, 63). The decreased Grb2 and c-Raf-1 may indicate that the MAPK cascade was de-emphasized in the MCF-7/5C cells, while the Akt cascade was more important. Also, decreased IRS-1 in conjunction with decreased IGF-1 receptor indicated a de-emphasis of the IGF-1 signaling to ERα as in the MCF-7/TAM2 tumors. The increased PKA catalytic subunit expression with a concomitant decrease in its regulatory subunit expression indicates that this may be an important pathway to promote ligand-independent ERα activity as discussed above (46, 61, 62). Moreover, an increase in ERα levels leads to increased ERα activity.

**Genes regulated by E$_2$ selectively in MCF-7/5C Tumors and Associated with Tumor Regression**
We examined gene expression profiles of wild-type MCF-7/E2 tumors and Phase II aromatase inhibitor-resistant MCF-7/5C tumors both treated with and without E$_2$. We then removed genes that were significantly changed in both cell lines, and focused on the genes selectively regulated by E$_2$ in the MCF-7/5C tumors.

**Differentially expressed genes that modulate cell adhesion and tight junctions in MCF-7/5C tumors (Figure 40)**
E$_2$ induced Snail1 and repressed claudins 3 and 4 in the Phase II aromatase inhibitor-resistant MCF-7/5C tumors, but E$_2$ did not regulate these genes in the wild-type MCF-7/E2 tumors. Claudins are involved in cell adhesion and tight junction formation; they are also critical in establishing and maintaining cell polarity (64). Interestingly, claudins 1, 3, and 4, ITGB1 (integrin β1), AF-6 (actin filament-binding protein or afadin), CSDA (cold-shock domain protein A), actin (β-actin, γ-actins 1 and 2), and caveolin-1 were all decreased in expression in MCF-7/5C tumors relative wild-type MCF-7/E2 tumors. Since these proteins all have roles in cell adhesion, their decreased expression in the MCF-7/5C tumors compared to the wild-type tumors indicates that the MCF-7/5C tumor cells would exhibit increased motility. Snail1 has previously been demonstrated to down-regulate claudins 3 and 4, and to promote epithelial to mesenchymal transition (EMT) transition (64). Hence, decreased expression of claudins 3 and 4 are markers of EMT. Therefore, E$_2$-induced Snail1 likely led to decreased expression of claudins 3 and 4, which may have driven the MCF-7/5C cells to undergo EMT, and in a cell type undergoing EMT, E$_2$-activated ERα may lead to apoptosis.

**Figure 40. Genes involved in cell adhesion and tight junctions as curated by GeneGo Metacore.** Description of the figure icons is given in the legend to Figure 35.

Elsewhere under Task 4a, we describe the gene expression microarray analysis of the wild-type MCF-7/WS8 and aromatase inhibitor-resistant MCF-7/5C cells in culture treated with and without $10^{-9}$ M $E_2$ over a 96 h time course. In those cell culture experiments, it was also found that $E_2$ preferentially induced Snail1 expression in a time-dependent manner in MCF-7/5C cells compared to wild-type MCF-7/WS8 cells **(Figure 41)**. Interestingly, it has been reported that stable ectopic expression of Snail1 in MCF-7 cells induces EMT by Snail1 binding the promoter of ERα and thereby switching off its expression, resulting in the MCF-7 cells becoming ERα negative (65). However, unlike the MCF-7 cells transfected with Snail1 as in the report (65), in our experiments in which $E_2$ induced endogenous Snail1 expression in MCF-7/5C cells, ERα expression was similarly downregulated to the same extent as in the wild-type MCF-7/WS8 throughout the time course **(Figure 42)**, and never became ERα negative. Hence it is provocative to speculate that opposing stimuli that promote epithelial differentiation via ERα as opposed to mesenchymal differentiation through Snail1 may lead to apoptosis in the MCF-7/5C cells.

We will functionally test whether Snail1 has a causative role in $E_2$-induced apoptosis. Snail1 will be depleted by siRNA transfection in MCF-7/5C and wild-type MCF-7 cells in culture, and the effects of Snail1 depletion on growth will be determined using a DNA-based cell proliferation assay. If depletion of Snail1 blocks $E_2$-induced apoptosis in the MCF-7/5C cells, we will continue to investigate the functional role of Snail1, and evaluate the utility of Snail1 as a biomarker in the clinical samples obtained from the clinical trial in this COE.

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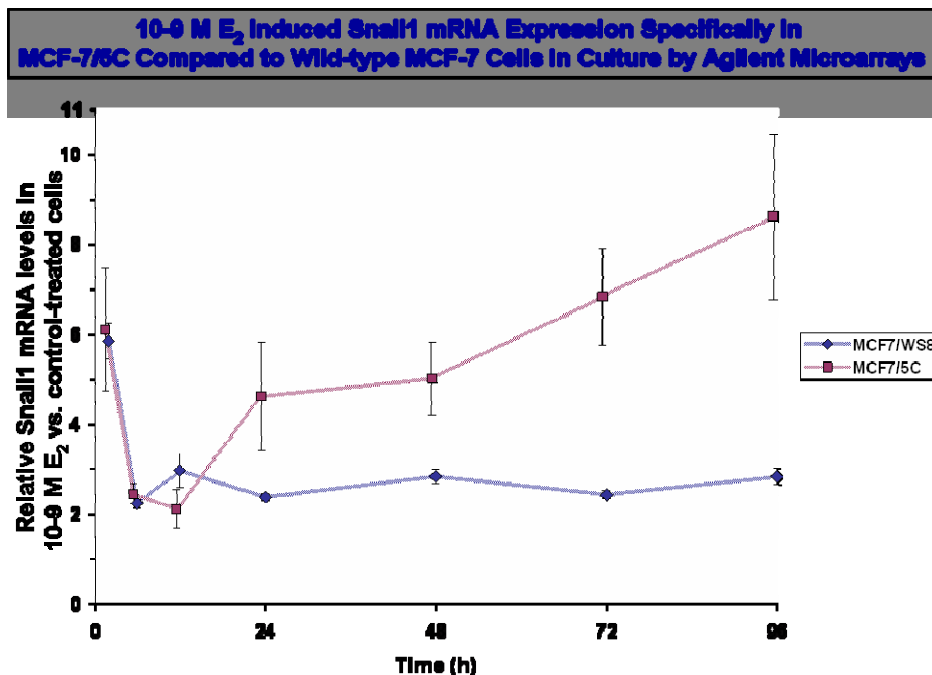
**Figure 41. Snail1 mRNA expression in $10^{-9}$M $E_2$-treated wild-type MCF-7/WS8 and aromatase inhibitor-resistant MCF-7/5C cells in culture.** RNA expression levels were determined using Agilent 4x44k human oligodeoxynucleotide microarrays and 6 replicate arrays per time point (2 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h).
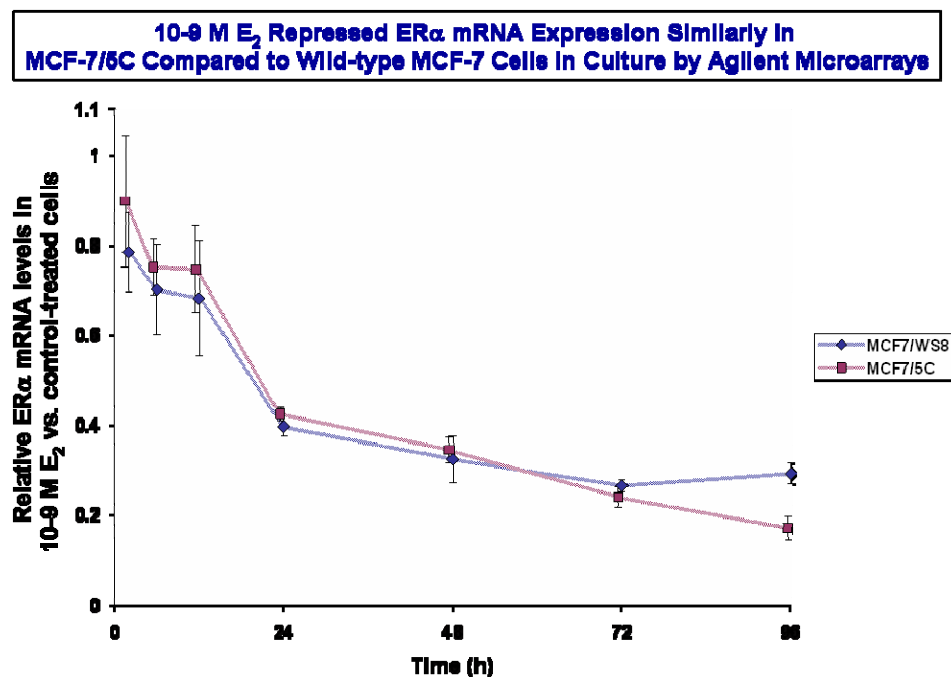


**Figure 42. ERα mRNA expression in $10^{-9}$M $E_2$-treated wild-type MCF-7/WS8 and aromatase inhibitor-resistant MCF-7/5C cells in culture.** RNA expression levels were determined using Agilent 4x44k human oligodeoxynucleotide microarrays and 6 replicate arrays per time point (2 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h).

**Potential Biomarkers**
Gene expression profiles of the antihormone resistant tumors versus the wild-type tumors to identify genes that showed the largest changes in expression. We found genes that may serve as biomarkers of antihormone resistance for either Phase I or Phase II disease, and we found genes that were selective for Phase I versus Phase II antihormone resistance.

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**Potential biomarkers of either Phase I or Phase II antihormone resistance (Figure 43)**

Genes consistently deregulated across all antihormone resistant tumors compared to wild-type MCF-7/E2 were identified. The genes that showed the largest consistent increases expression were IER3 (immediate early response 3) and TRPM7 (transient receptor potential cation channel, subfamily M, member 7), while some of the genes that showed the largest consistent decreases were the ERα target genes PGR (progesterone receptor), NRIP1 (RIP140), IGF1R (insulin-like growth factor 1 receptor), and GREB1.  These genes have potential to serve as biomarkers of either Phase I or Phase II antihormone-resistant breast cancer, and thus may have broad utility in selecting breast cancer treatment course.
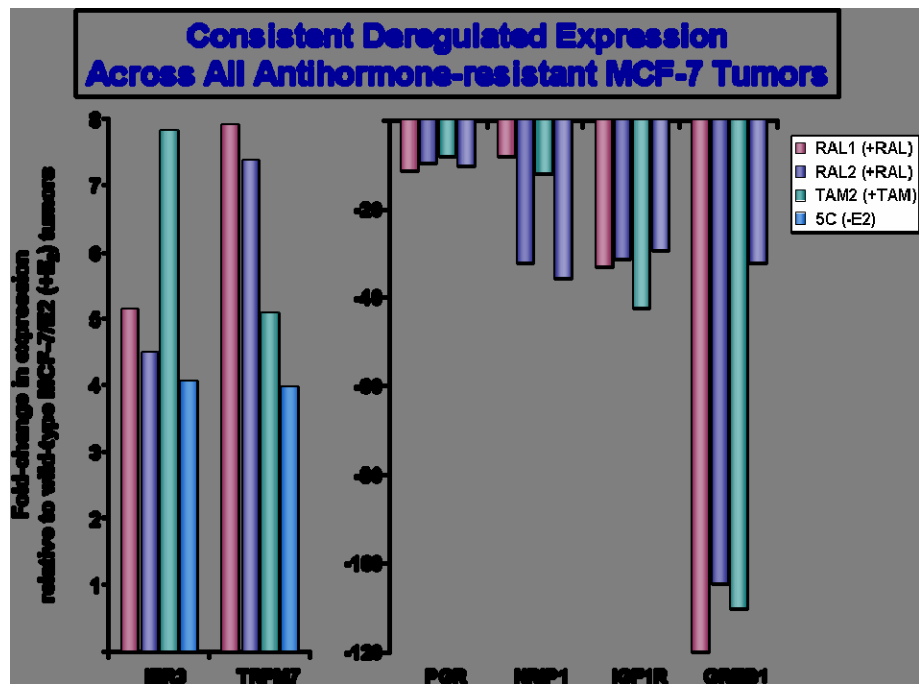


**Figure 43. Potential biomarkers of either Phase I or Phase II antihormone resistance.**

**Potential biomarkers selective for Phase I versus Phase II antihormone resistance (Figure 44)**

Genes that were selective deregulated in Phase I versus Phase II were identified; PCDH7 (protocadherin 7), S100A8 (S100 calcium binding protein A8, calgranulin A), and S100A9 (S100 calcium binding protein A9, calgranulin B) were selectively increased in Phase I MCF-7/RAL tumors; while, S100A9, S100P (S100 calcium binding protein P), COL5A2 (collagen, type V, alpha 2), and KRT17 (keratin 17) were selectively decreased in Phase II tumors. Expression levels of these genes will be evaluated as biomarkers of response to estrogen therapy in the clinical trial in this COE.

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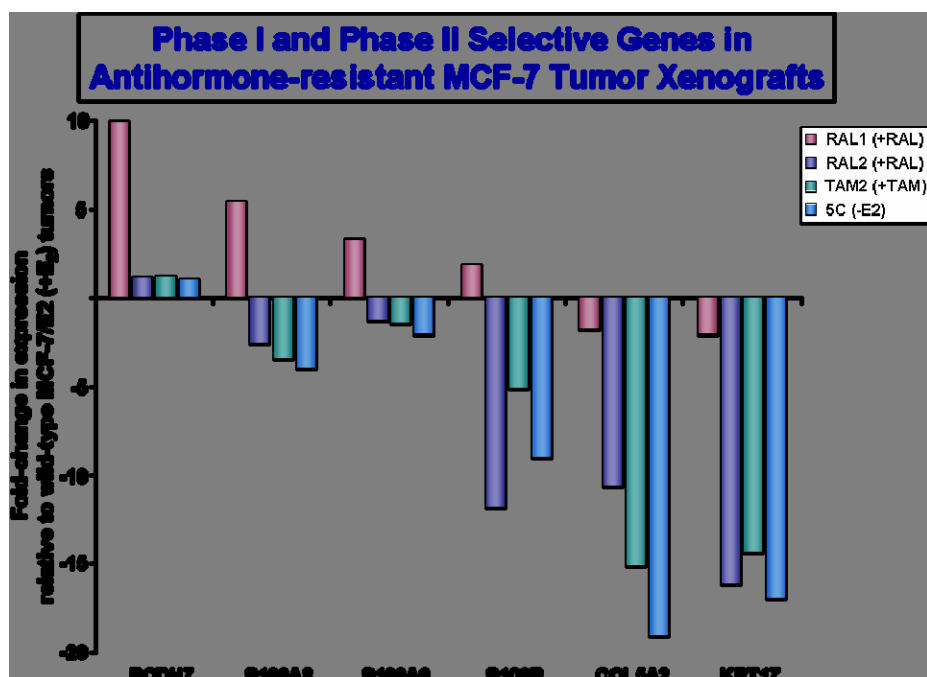
**Figure 44. Potential biomarkers selective for Phase I versus Phase II antihormone resistance.**

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**TASK 4 (TGen/Cunliffe): To analyze E$_2$-induced survival and apoptotic pathways using gene arrays and siRNAs**

**Overarching scheme of experiments in this task:** Array-based expression profiling of all *in vitro* and *in vivo* models generated under **Task 2** will be employed to identify genes and pathways associated with survival and apoptosis mechanisms.

**Task 4a.  Catalogue the transcriptional response using array-based expression profiling.**

**Task 4b.  Identify regulatory networks for pathways indicative of differential responses to E$_2$.**

**Task 4c.  Interrogate pathways of endocrine resistance using high throughput RNA interference (HT-RNAi)**

Here we report work completed on Tasks 4a - 4c at The Translational Genomics Research Institute site during year 2 of this COE. This involves time course analysis of E$_2$-regulated gene expression in the *in vitro* models MCF-7:WS8 and MCF-7:5C cells over a 96 h time frame.

**WORK ACCOMPLISHED - TASK 4A AND 4B**

**Table 5** below summarizes the work performed on cell line Modules 1 and 2 as outlined in the original proposal.  The overarching goal is to analyze global patterns of E$_2$-mediated gene regulation in wild-type (MCF-7:WS8) compared phase II models of endocrine resistance which undergo apoptosis following exposure to E$_2$.  Gene expression profiling on the first module (including MCF-7:WS8 and MCF-7:5C cells with and without estrogen induction) have been completed.  This included time points of 2h, 6h, 12h, 24h, 48h, 72, and 96h with 6 replicates at each time point collected with no E$_2$, and 6 replicates collected at each time point following E$_2$ induction.

| | Workflow. Task 4b | Month (Sep 2007-Aug 2008) |
|---|---|---|
| | | S O N D J F M A M J J A |
| 1 | 42 Hybridizations performed for MCF7:WS8 +/- E2 | |
| 2 | 42 Hybridizations performed for MCF7:5C +/- E2 | |
| 3 | Data Feature extraction and QC macros performed on 84 hybridizations | |
| 4 | Hybridization Quality Control | |
| 5 | Database assembly | |
| 6 | Data analysis I (template based) | |
| 7 | Data analysis II (distance based) | |
| 8 | Data analysis III (time point delta difference based "inflection" analysis) | |
| 9 | Collaborative analysis of Phase I-III data (TGen and FCCC) | |
| 10 | Receipt and inventory of MCF7:2A long time course TRIzol lysate 2/28/08 | |
| 11 | Receipt and inventory of MCF7:2A short time course TRIzol lysate 3/4/08 | |
| 12 | Preparation and QC of long time course MCF7:2A RNA (9 days, 108 RNA preps) | |
| 13 | Preparation and QC of short time course MCF7:2A RNA (96h, 84 RNA preps) | |
| 14 | Final spectrophotometry and aliquot RNA to be returned to FCCC | |
| 15 | RNA Shipped to FCCC 7/28/08 for Q-RTPCR analysis | |
| | **Key** | |
| | Blue denotes Module 1 TGen workflow (MCF7:WS8 and MCF7:5C cells) | |
| | Red denotes Module 2 TGen workflow (MCf7:2A cells) | |

**Table 5.  Summary of work performed at TGen under Task 4a and 4b, September 2007-August 2008.**

Eighty four individual RNA extractions were performed for the MCF-7:WS8 time course and 84 from the MCF-7:5C time course.  Forty two separate 2-color gene expression microarrays were performed for the MCF-7:WS8 time course (using time point-matched RNAs as reference samples).  The same procedure was followed for the MCF-7:5C cells. To reduce cross-experimental error due to the significant time frame in which these hybridizations needed to be batched, hybridizations were performed such that at least one replicate from each time point was included in all batches of arrays.  For example, the first replicate from all time points (2 through

96 h) were hybridized in batch 1, etc. All 84 hybridizations met all array-based quality control statistics. Data was preprocessed prior to analysis by removing Q/C probes, median intensity values were used for replicate array control probes. This reduced the raw data from 44,000 to ~41,000 features per array.

**Data analysis**

Three discrete methodologies have been applied to interrogate the temporal gene expression data generated from MCF-7:WS8 and MCF-7:5C cells in order to identify gene regulatory networks and molecular drivers of the apoptotic response to estrogen in MCF-7:5C cells. Results from the three data analysis methodologies will be presented separately. The information from all three methodologies is currently being integrated in collaboration with additional analyses being performed on the same dataset at FCCC.

The three temporal data analysis methodologies are as follows:

        Temporal Data analysis methods:
        • Template based
        • Distance based
        • Inflection based

**METHOD 1 - Temporal data analysis:  Template-Based**

Template based analysis defines a correlation between temporal patterns of gene expression profiles to a series of user predefined temporal templates.
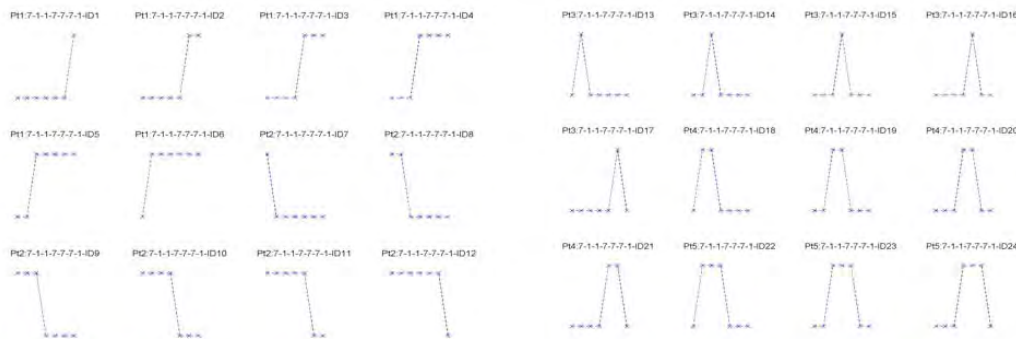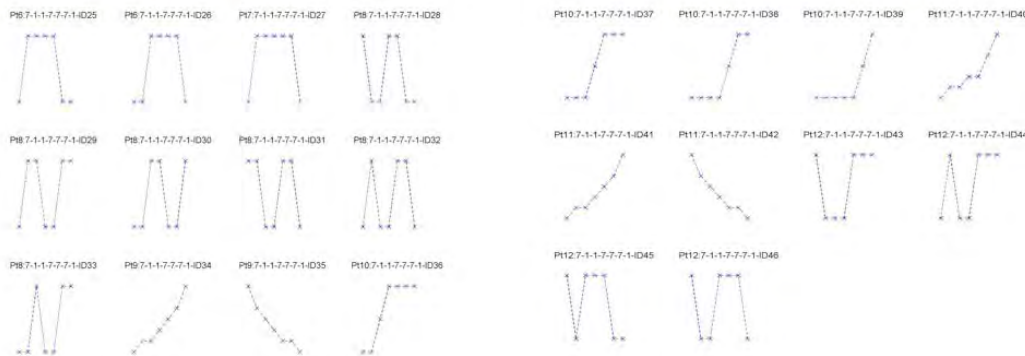


**Figure 45.  Profiles of 46 prefixed templates to cover most gene expression pattern changes over the time intervals.** The analytical goal is to find genes with a high a Pearson correlation to a given template in one cell type but no correlation or ant-correlation in the other cell type.

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**NFKBIA**

A₂3ₚ106002:NFKBIA ||Low-5C-High-WS8 [PtID:35 Corr>= -0.51; >= 0.94]

| High-WS8-Low-5C (Corr >= 0.9 & <= 0.1) | | |
|---|---|---|
| PatternType | PatternID | NumGenes |
| Pt9 | 35 | 20 |
| Pt10 | 38 | 20 |
| Pt11 | 42 | 20 |
| Pt10 | 37 | 19 |
| Pt2 | 9 | 12 |
| Pt9 | 34 | 12 |
| Pt11 | 41 | 12 |
| Pt1 | 2 | 11 |
| Pt1 | 3 | 9 |
| Pt2 | 10 | 5 |
| Pt11 | 40 | 5 |
| Pt10 | 39 | 2 |
| Pt1 | 4 | 1 |
| Pt2 | 11 | 1 |
| Pt10 | 36 | 1 |

**Figure 46. Example from template-based analysis.** The gene NFKBIA shows a high match to template pattern #35 in MCF-7:5C cells (red), but not in MCF-7:WS8 cells (green). The x-axis is a plot of data correlation across the 7 time points collected. 1 standard deviation bounds (of replicates) are shown as dotted lines.

**METHOD 2 - Temporal data analysis: Distance Based**
This analysis interrogates patterns of gene expression within a dataset based on relative intensity value change, and is not limited to a fixed or predefined pattern. Using intensity data from each color channel, a distance metric was computed across the time points for a gene $i$ between the treatments (presence or absence of $E_2$) in a given cell type (MCF-7:5C or MCF-7:WS8). Intensity value from each color channel were normalized to the median microarray chip intensity value and log2 transformed to allow direct inter-array comparison. The extent of change computed between any two experimental conditions was computed using Euclidean distance:
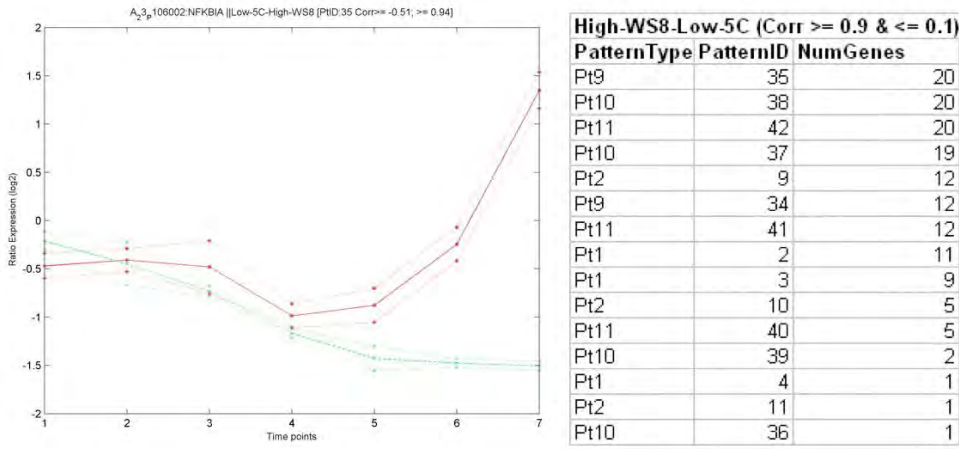
$$D = [G_i(plus\ E_2) - G_i(no\ E_2)]$$

$$\Rightarrow \quad D = \frac{1}{n_t} \sum_{k=1}^{n_t} G_{i:With-E2}(k) - G_{i:No-E2}(k)$$

The distance measure could increase, decrease or show no change when two experimental conditions are compared. The distance measure was computed for all possible pairs of replicates (n-15) within the 4 groups of data (MCF-7:5C, MCF-7:5C + $E_2$, MCF-7:WS8, MCF-7:WS8 + $E_2$). The distance cutoff can be arbitrarily set. For our preliminary analyses we defined the cutoff at ±1σ. **Figure 47** shows the variation in the distribution of differences among all possible pairs of replicates within each group (solid blue line) with 1σ (0.8512, -0.8429) and 2σ (1.698, -1.69) cut off lines (blue and red lines respectively). A difference level greater than or equal to ($\mu+1\sigma$) is defined as 'Positive (+) Change,' any difference level less than or equal to ($\mu-1\sigma$) is defined as 'Negative (-) Change' while any difference between them is defined as 'Zero (0) or No-Change.' As a conservative measure we repeated the analysis on a computed weighted average among time point replicates (any sample over ±σ, was removed and mean computed & deviation computed on rest) for each gene across experiments. The distribution of individual samples is shown graphically in **Figure 48** as dotted red and green lines in the graph. Red (positive) if $D \geq (\mu+1\sigma)$, Green (negative) if $D \geq (\mu-1\sigma)$ and Blue (zero) if $D > (\mu-1\sigma)$ & $D < (\mu+1\sigma)$. **Table 6** lists the distribution of significantly changed genes using the distance-based method.

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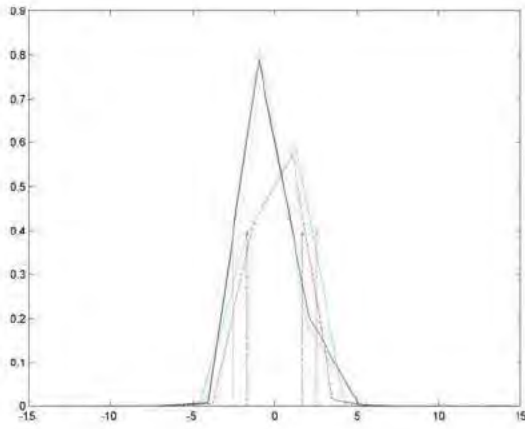
**Figure 47**. Variation in the distribution of differences among all possible pairs of replicates for MCF-7:WS8 and MCF-7:5C groups using the distance-based analysis metric. Solid blue line shows the overall distribution of differences among all possible replicates within each group. Red (positive) if $D \geq (\mu+1\sigma)$, Green (negative) if $D \geq (\mu-1\sigma)$ and Blue (zero) *if* $D > (\mu-1\sigma)$ *&* $D < (\mu+1\sigma)$.



A$_2$3$_p$147431 LYN || 5C-D(No-E2,E2)-Pos-1sig–WS8-Neg [Dist1 0.916, Dist2 -1.168, Ratio 0.841]

5C (No-E2)  5C(W-E2)  WS8 (No-E2)  WS8 (W-E2)
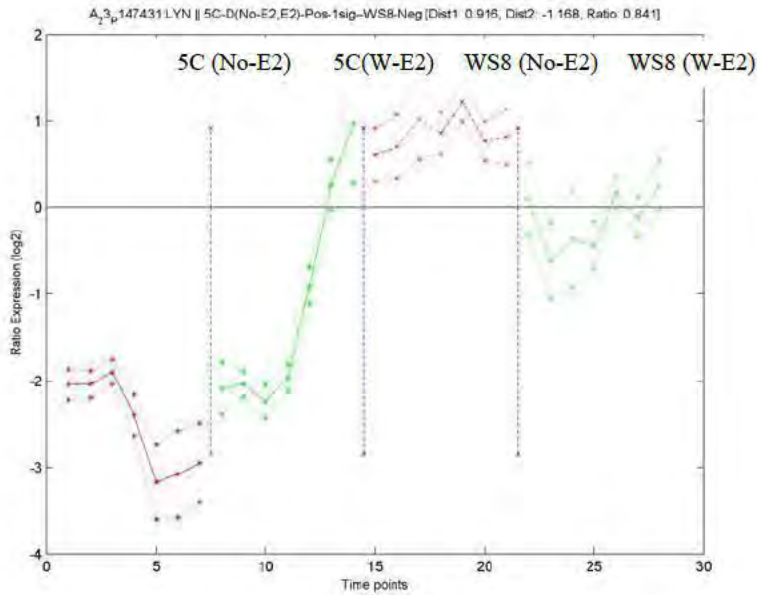
**Figure 48.** Example from distance-based analysis. Distance metric for a given gene in the presence versus absence of E$_2$ is positive in MCF-7:5C cells and negative in MCF-7:WS8 cells.

**Distance metric computed using all replicates**

| SN | Distance Type | D( WS8-No-E2, WS8-W-E2) with $(\mu\pm1\sigma)$ | | |
|---|---|---|---|---|
| | (All replicate) | Positive (+) | Zero(0) | Negative(-) |
| 1 | D( 5C-No-E2, 5C-W-E2) | Positive (+) 243 | 146 | 2 |
| 2 | | Zero(0) 408 | 30,708 | 454 |
| 3 | | Negative(-) 0 | 162 | 326 |

**Distance metric computed using weighted average of all replicates**

| SN | Distance Type | D( WS8-No-E2, WS8-W-E2) with $(\mu\pm1\sigma)$ | | |
|---|---|---|---|---|
| | (Weighted average) | Positive (+) | Zero(0) | Negative(-) |
| 1 | D( 5C-No-E2, 5C-W-E2) | Positive (+) 487 | 555 | 3 |
| 2 | | Zero(0) 912 | 36,994 | 1091 |
| 3 | | Negative(-) 0 | 432 | 526 |

**Table 6. Summary of number of statistically significant genes identified using the distance-based method.** The top table computed using all replicates and the bottom table computed using the more

conservative weighted average of all replicates.

**METHOD 3 - Temporal data analysis: Inflection Based**
A Custom data analysis methodology was developed for this study specifically to identify individual driver or trigger ('inflection') genes that show a dramatic change in expression at a time point that mirrors a dramatic change in cell biological behavior (such as the apoptotic response of MCF-7:5C cells at approximately 48h post $E_2$-treatment. The delta differences are defined as the change in expression of a given gene between time points with the starting point serving as the initial reference. Genes identified by this method are also considered with respect to wild-type MCF-7:WS8 cells that do not show the same apoptotic response to $E_2$ at a given time point. Using log2 normalized values, delta differences are progressively computed across the entire time course for each cell line being interrogated. A gene at a time point is considered an 'inflection' if the delta difference is $3\sigma$ greater than the computed experimental variation. Each time interval for a given gene is assigned a flag of '1' if inflected otherwise a zero "0" to facilitate hypothesis generation and prioritization for subsequent validation.

$$D = [\text{Delta}G_i(\textit{With-E}_2) - \text{Delta}G_j(\textit{No-E}_2)]$$

$$\Rightarrow deltaD = \frac{1}{n_t} \sum_{k=1}^{n_t} DeltaG_{i:With-E2}(k) - DeltaG_{i:No-E2}(k)$$
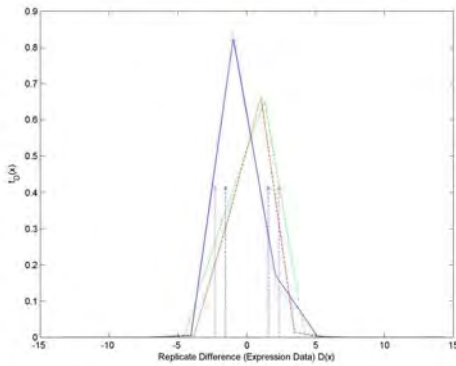


**Figure 49.** Distribution of differences among all possible experimental replicates within each group (blue solid line) with $1\sigma$ (0.7695, -0.7695 ) & $2\sigma$ (1.5597, -1.5597) cutoff lines in blue and red respectively. Positive and negative inflection gene distributions are denoted by the red and green dotted lines.

**Finding Inflected Genes.** It is widely believed that genes that show sudden change in expression value observed over time may be the molecular trigger for biological change (apoptosis/ increased growth) and related to cell mechanisms being investigated. In this study, $E_2$ induces apoptosis in Phase II resistant cells (such as MCF-7:5C) while it induces proliferation in MCF-7:WS8 cells. Our interest is to find the genes that show a sudden change in its mRNA level or gene-expression as progressed over time. This will help us to identify pathways they may be associated specifically with estrogen-induced apoptosis.

To briefly describe the methodology, the log2 normalized data is first converted to a level difference data by progressively computing the expression difference from its previous time observation. We refer to this as 'delta' data for 1 to 7 time points, where start time will be Zeros at 2hrs (reference), second (6hrs -2hrs), third (12hrs - 6hrs), fourth (24hrs – 12hrs), fifth (48hrs – 24hrs), sixth (72hrs – 48hrs), seventh (96hrs – 72hrs). This is repeated independently for all the replicates.

Our goal is to find inflections that are pronounced in each of experiment (5C-No-$E_2$, 5C-W-$E_2$, WS8-No-$E_2$, WS8-W-$E_2$). Where a delta time point (1 to 7 meaning 2hrs to 96hrs) for a gene will be assigned '1'(inflected) otherwise '0' (not inflected) only if the delta difference is greater than the confidence level set by the variation among the experimental replicates **(Figure 49)**. An inflection can occur in any time point but has to be limited to one of the experimental conditions. Genes are identified using this methodology that show inflections with or with out introduction of $E_2$ (see **Table 7** for a data summary). Lastly, genes show consistent inflections in any of the time points are sorted by total number of inflections (sum) across experiments in ascending fashion.

The same analysis as described above was repeated with 'weighted delta data' where outlier delta data points ($\mu\pm1\sigma$) were omitted before computing the weighted average. This process results in identification of greater numbers of inflected genes. **Table7** gives a frequency on the genes identified using delta difference and weighted delta difference. As indicated by the summary in **Table 7**, very few delta differences are observed for the No-$E_2$ experiments (as expected) as these are control cell lines untreated. Significant numbers of inflected genes are identified as inflected following $E_2$ induction, and primarily occurring at one time point, suggesting strong and transient transcriptional flux at a discrete time-point.

| | Inflection Table: Delta difference data (3σ cutoff) | | | | | |
|---|---|---|---|---|---|---|
| | | **Number of Inflections** | | | | |
| **No** | **Experiments** | **1** | **2** | **3** | **>4** | **Total** |
| 1 | 5C(No-E2) Only | 0 | 0 | 0 | 0 | 0 |
| 2 | 5C(W-E2) Only | 12 | 0 | 0 | 0 | 12 |
| 3 | WS8(No-E2) Only | 0 | 0 | 0 | 0 | 0 |
| 4 | WS8(W-E2) Only | 3 | 0 | 0 | 0 | 3 |
| | **Any Experiment** | 16 | 0 | 0 | 0 | 16 |

| | Inflection Table: Weighted Delta difference (3σ cutoff) | | | | | |
|---|---|---|---|---|---|---|
| | | **Number of Inflections** | | | | |
| **No** | **Experiments** | **1** | **2** | **3** | **>4** | **Total** |
| 1 | 5C(No-E2) Only | 0 | 4 | 0 | 0 | 4 |
| 2 | 5C(W-E2) Only | 44 | 0 | 0 | 0 | 44 |
| 3 | WS8(No-E2) Only | 29 | 1 | 0 | 0 | 30 |
| 4 | WS8(W-E2) Only | 71 | 2 | 0 | 0 | 73 |
| | **Any Experiment** | 148 | 12 | 0 | 0 | 160 |

**Table 7. Frequency of inflected genes across this dataset at 3σ.** Columns denoting number of inflections indicates genes inflected in 1, 2, 3 or $\geq$ 4 out of a possible 7 delta distance measures for each experiment.
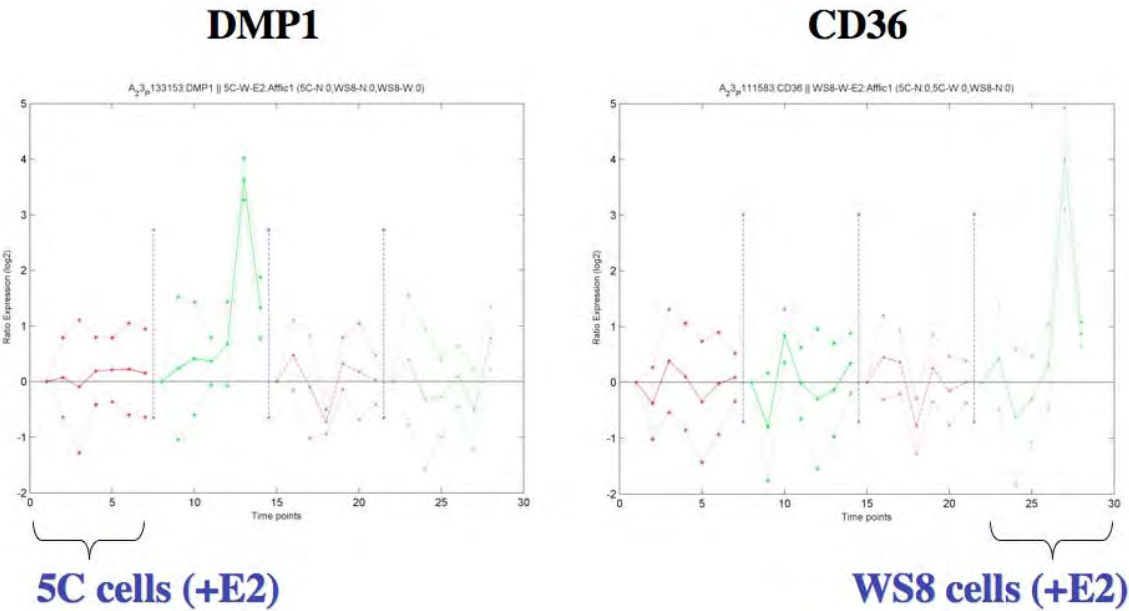


**Figure 50. Two of the top gene picks identified by the delta difference methodology.**

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**Pathway analysis of all inflected genes (using Ingenuity Pathway Analysis software).** Genes identified with an inflection in WS8 or MCF-7:5C cells were examined for mechanistic network association using ingenuity software. The output files are shown in **Figures 51** and **52**. The top molecular and cellular function associated with MCF-7:5C cells was cell death, as expected. Our preliminary analysis of MCF-7:5C cells suggests that sensitivity to estrogen may be due to altered regulation of TNF, NFκB and/or AKT pathway activities. This is yet to be confirmed and validated bioinformatically and experimentally.
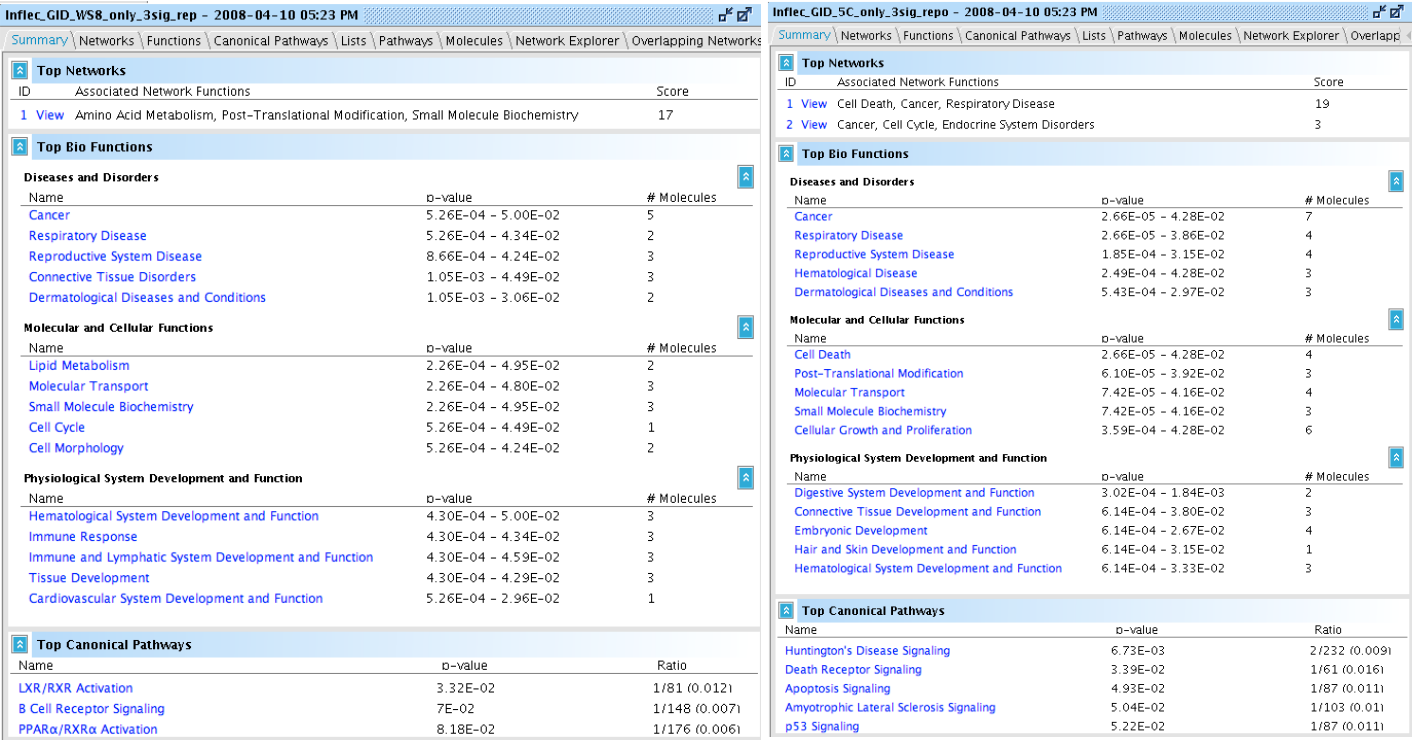


**Figure 51. Ingenuity pathway analysis summary of inflected genes in MCF-7:WS8 and MCF-7:5C cells.** The MCF-7:WS8 data on the left show expected ontologies associated with cell cycle progression and cell metabolism. On the right, E$_2$ inflected genes are associated with cell death.
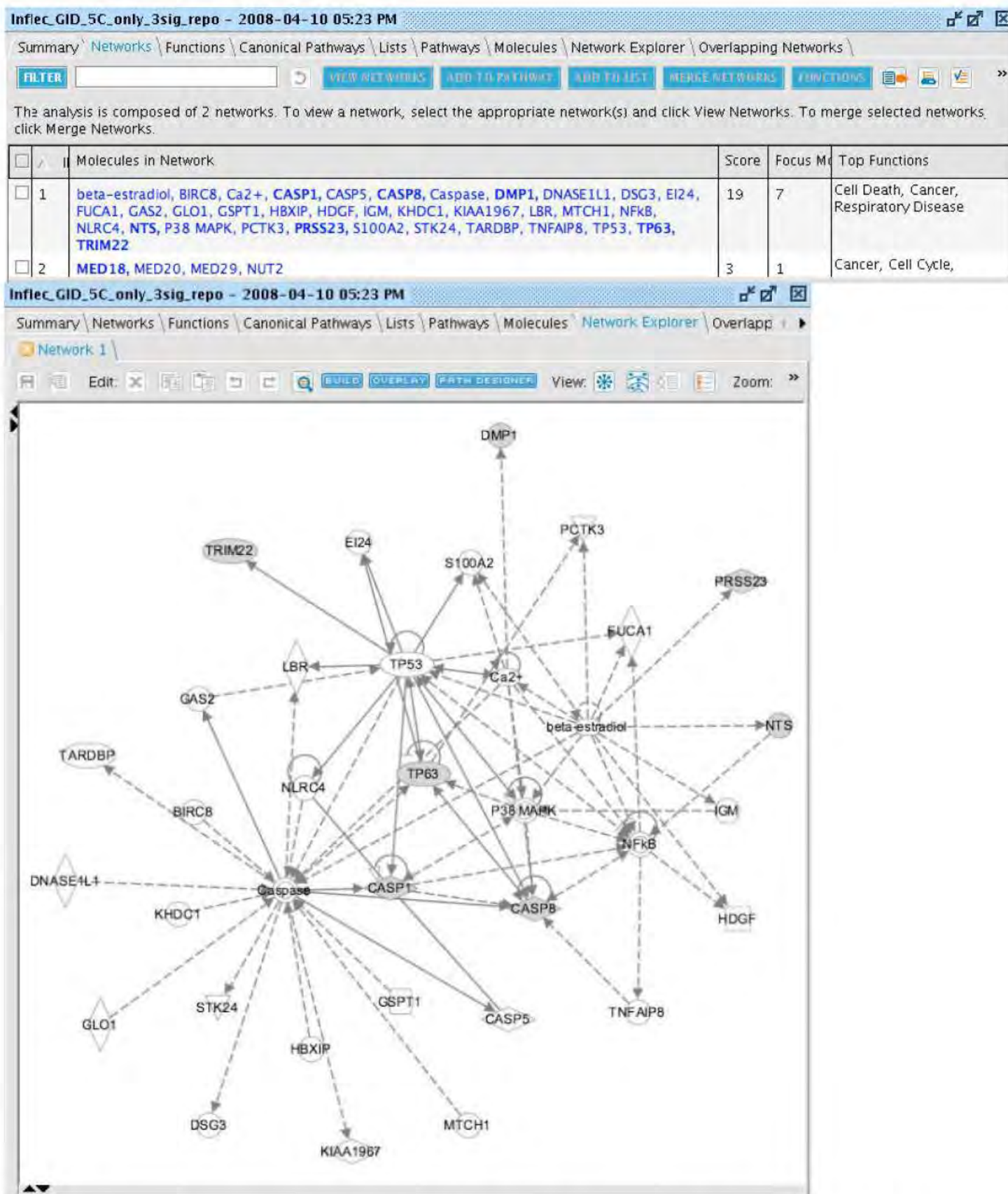
**Figure 52. Ingenuity pathway analysis in MCF-7:5C cells.** The top panel shows genes in the top scoring

network (cell death, cancer), and genes in bold are positive from our gene inflection analysis. The corresponding network is drawn in the lower panel.

## WORK ACCOMPLISHED – TASK 4C

**Optimization of siRNA transfection in MCF-7:2A and MCF-7:5C cell lines.** As part of Assay Development for High-throughput RNAi (HT-RNAi) screening, we have initiated siRNA transfection optimization on MCF-7:2A and MCF-7:5C cell lines. Our transfection reagent screening test is done under HT-RNAi assay conditions, which includes seeding cells in 384-well plates and growth for 96 hours. Both cell lines were treated under twenty-one transfection conditions using a panel of four transfection reagents and five lipid:siRNA (vol:wt) ratios of each reagent. The siRNA used for transfection included a non-silencing siRNA, a positive control lethal siRNA and a no siRNA treatment. Results are shown in **Figure 53** and indicate that the transfection reagent RNAiMax worked well for both cell lines at ratios of 4:1 up to 10:1. We are choosing RNAiMax at a ratio of 6:1 for future transfection experiments.
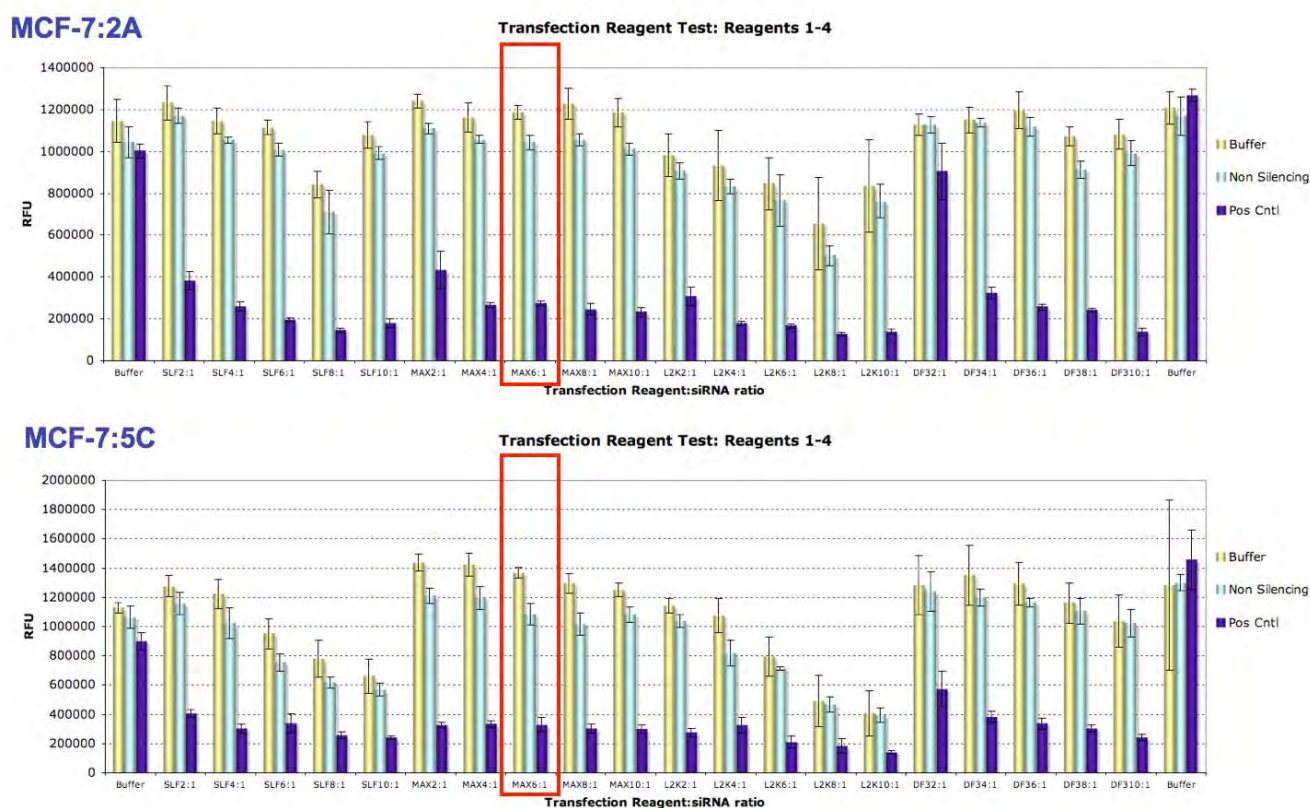


**Figure 53. Transfection Optimization of MCF-7:2A and MCF-7:5C cells.** MCF-7:2A and MCF-7:5C were transfected with either Non-silencing siRNA (neg. control), Positive control siRNA (toxic) or no siRNA (Buffer) at varying reagent:siRNA ratios (vol/wt). Several reagents were able to efficiently transfect the cells with little toxicity. The selected reagent for both cell lines was RNAiMax at a 6:1 reagent:siRNA ratio which is highlighted by the red box.

**Response of MCF-7:2A and MCF-7:5C cells to $E_2$ treatment under HT-RNA assay conditions.** The MCF-7:2A and MCF-7:5C cell lines were tested for their response to $E_2$ under HT-RNAi conditions. MCF-7:2A and MCF-7:5C cells were seeded at 1000 cells per well in 384-well plates and treated with varying doses of $E_2$ at 24 h. After 72 h of $E_2$ exposure, proliferation was assessed by using Cell Titer-Glo (Promega). Both MCF-7:2A and MCF-7:5C cells showed decrease proliferation at 72 h for the higher doses tested **(Figure 54)**. At the target

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dose of 1 nM, the MCF-7:5C cells showed about a 15% decrease in cell while the MCF-7:2A cells did not show a decrease. Previous results indicate that the MCF-7:2A cells begin undergoing apoptosis at day 7 after 1 nM $E_2$ exposure and thus to adapt these cells to RNAi screening, we plan to examine both longer assay times and higher $E_2$ concentrations. For the MCF-7:5C cells, current assay conditions suggest that these cells can go into HT-RNAi validation screening using small siRNA libraries.
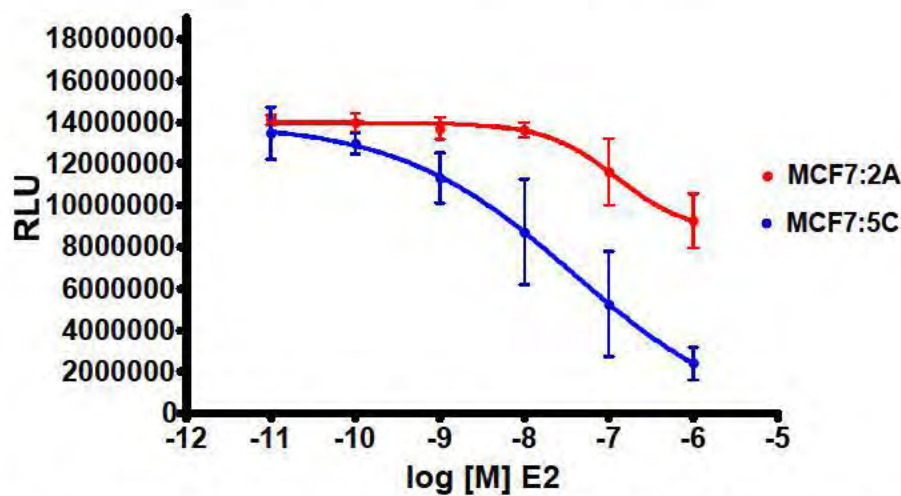


**Figure 54. Effect of estradiol treatment of MCF-7:2A and MCF-7:5C cell proliferation.** MCF-7:2A and MCF-7:5C cells were treated with varying doses of estradiol under HT-RNAi conditions. After 72 estradiol exposure, cell number was determined using Cell Titer Glo (Promega).

# KEY RESEARCH ACCOMPLISHMENTS

**Administration**
- Established an effective multi-institutional collaborative research program to link the biology of estrogen action (growth or apoptosis) with proteomics and genomic changes. Data can now be catalogued, stored and evaluated electronically.

**Task 1 (FCCC/Goldstein, Swaby)**
- The clinical trial to evaluate dose de-escalation of estrogen (Estrace) to reverse antihormone resistance in patients treated exhaustively with antihormone therapy has been approved by the FCCC internal review board, Astra-Zeneca Pharmaceuticals, and the Department of Defense.
- This trial is now screening and enrolling patients at FCCC.

**Task 2a (FCCC/Jordan, Ariazi)**
- Generated protein lysates for proteomics of MCF-7/WS8, MCF-7/5C, and MCF-7/2A cells.
- Generated RNA (TRIzol) lysates for gene expression microarrays of MCF-7:2A cells treated with $E_2$ over a short time course (96 h) and a long time course (9 days). The RNA samples have been fully quality control to have been derived from cells exhibiting the expected growth response to E2, and quality controlled for RNA integrity and gene expression markers.

**Task 2b (FCCC/Jordan, Lewis-Wambi)**
- Discovered that the MCF-7: 5C and 2A cells have different time courses for their apoptotic response to $E_2$. Further studies can now compare time courses in more detail and discover the reason for the potential resistance to estrogen and discovered that the glutathione synthesis inhibitor BSO can enhance $E_2$ induced apoptosis.
- Discovered that the invasion protein CEACAM6 is elevated in cells and confers increased invasive potential in MCF-7:5C cells.

**Task 2b (FCCC/Jordan, Sengupta)**
- Discovered that the antiapoptotic estrogen regulated protein Bcl-2 is actually regulated by another estrogen regulated protein XBP-1.

**Task 3 (GU/Riegel and Wellstein)**
- Conducted IPs of AIB1 and Tyr-phosphorylated proteins and identified co-IPed interacting proteins using MS in MCF-7, MCF-7:5C and MCF-7:2A cells treated with and without $E_2$.
- Discovered that RNA metabolism and transcription related proteins are major functional groups interacting with AIB1 in $E_2$ treated MCF-7/5C cells.
- Discovered that G-protein coupled receptor signaling pathways are associated with $E_2$-induced MCF-7/5C cell apoptosis.

**Task 4a-4b (FCCC/Jordan, Ariazi; in collaboration with TGen/Cunliffe)**
- Completed a preliminary gene expression microarray analysis of antihormone-resistant *in vivo* breast cancer tumor models.
- Discovered that the corepressor RIP140 (receptor interacting protein 140; NRIP1, nuclear receptor interacting protein 1) was significantly decreased in all the antihormone-resistant models compared to wild-type MCF-7/E2 tumors, indicating deregulation of ligand-dependent activation of ERα.
- Discovered that G-proteins were differentially regulated in the antihormone resistant tumors compared to wild-type MCF-7/E2 tumors, indicating activation of non-genomic ERα signaing in the antihormone resistance.

- Discovered genes selectively associated with different models of antihormone resistance that all eventually target ERα for ligand-independent activation.
- Discovered that Snail1, an important mediator of epithelial-to-mesenchymal transition, may be involved in $E_2$-induced apoptosis.
- Identified potential biomarkers of Phase I and Phase II antihormone resistance that are good candidates for evaluation in biopsy samples from patients in the Estrace trial.

**Task 4a-4c (TGen/Cunliffe)**
- Completed gene expression microarrays of MCF-7:WS8 and MCF-7:5C cell lines induced with estrogen over a 96 h time course.
- Completed RNA extraction and quality control of MCF-7:2A cells treated with $E_2$ over a long time course (9 days) and a short time course (96h), ready to be profiled.
- Developed and applied three different statistical methodologies to characterize gene expression changes consistent with rapid estrogen-induced apoptosis of MCF-7:5C cells. Preliminary data suggests deregulation of TNF, NFκB and AKT signaling pathways may be playing a role in sensitivity to $E_2$-induced apoptosis.
- Confirmed that MCF-7:5C and MCF-7:2A have different time courses for their apoptotic response to estrogen. High throughput RNAi analysis on the MCF-7:5C cells is currently in progress.

## REPORTABLE OUTCOMES

## Publications

Ariazi EA, Leitao A, Oprea, TI, Chen B, Louis T, Bertucci AM, Sharma CG, Gill SD, Kim HR, Shupp HA, Pyle JR, Madrack A, Donato AL, Cheng D, Paige JR, Jordan VC. Exemestane's 17-hydroxylated metabolite exerts biological effects as an androgen. *Mol Cancer Ther* 2007: 6(11):2817-27.

Jordan VC. New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer. *Steroids* 2007: 72(13):829-42.

Jordan VC. Tamoxifen or raloxifene for breast cancer chemoprevention: a tale of two choices—point. *Cancer Epidemiol Biomarkers Prev* 2007: 16(11):2207-9.

Jordan, VC. Estrogen receptors in BRCA1-mutant breast cancer: now you see them, now you don't. *J Natl Cancer Inst* 2007: 99(22):1655-7.

Jordan VC, Lewis-Wambi J, Kim H, Cunliffe H, Ariazi E, Sharma CG, Shupp HA, Swaby R. Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients. *Breast* 2007: 16:S105-13.

Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. *J Clin Oncol* 2007: 25(38):5815-24.

Patel RP, Sharma CG, Jordan VC. Optimizing the antihormonal treatment and prevention of breast cancer. *Breast Cancer* 2007: 14(2):113-22.

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Patel RR, Pyle JR, Jordan VC. The current clinical indications for fulvestrant. *Clinical Updates in Cancer* 2007: 2:4-6.

Jordan, VC. The 38th David A. Karnofsky lecture: the paradoxical actions of estrogen in breast cancer – survival or death? *J Clin Oncol* 2008: 26(18):3073-82.

Jordan, VC. Tamoxifen: catalyst for the change to targeted therapy. *Eur J Cancer* 2008: 44(1):30-8.

Lewis-Wambi JS, Cunliffe HE, Kim HR, Willis AL, Jordan, VC. Overexpression of CEACAM6 promotes migration and invasion of oestrogen-deprived breast cancer cells. *Eur J Cancer* 2008: 44(12):1170-79.

Sengupta S, Jordan VC. Selective estrogen modulators as an anticancer tool: mechanisms of efficiency and resistance. *Adv Exp Med Biol* 2008: 630:206-19.

Swaby RF, Jordan VC. Low-dose estrogen therapy to reverse acquired antihormonal resistance in the treatment of breast cancer. *Clin Breast Cancer* 2008: 8(2):124-33.

Ariazi EA and Jordan VC. Estrogen Receptors as Therapeutic Targets in Breast Cancer. In: Methods and Principles of Medicinal Chemistry (E. Ottow and H. Weinmann, eds.) Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim, Berlin, Germany, pp. 127-199, 2008.

Balaburski G and Jordan VC. Practical progress in the chemoprevention of breast cancer with selective estrogen receptor modulators (SERMs). In: Breast Cancer: Prognosis, Treatment and Prevention, 2nd edition (Jorge R. Pasqualini, ed.) Informa Healthcare, USA, Inc., New York, NY, pp 253-266, 2008.

Jordan VC. A century of deciphering the control mechanisms of estrogen action in breast cancer: the origins of targeted therapy and chemoprevention. *Cancer Res* 2008 (in press).

Oseni TS, Patel RP, Pyle JR, Jordan VC. Selective estrogen receptor modulators and phytoestrogens. *Planta Medica* 2008 (in press).

Oh AS, Lahusen JT, Chien CD, Fereshteh MP, Zhang X, Dakshanamurthy S, Xu J, Kagan BL, Wellstein A, Riegel AT. Tyrosine phosphorylation of the nuclear receptor coactivator AIB1/SRC-3 is enhanced by Abl kinase and is required for its activity in cancer cells. *Mol Cell Biol.* 2008 Sep 2 (Epub ahead of print).

Lewis-Wambi JS, Kim HR, Wambi C, Patel R, Pyle J, Klein-Szanto AJ, Jordan VC. Buthionine sulfoximine sensitizes hormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res 2008* (submitted).

## Abstracts

*Abstract #6348 was published in the 2007 Proceedings of the American Association for Cancer Research Special Conference in Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications.*
**Comparative Global Gene Expression Profiling to Identify Unifying and Selective Pathways Involved in Tamoxifen, Raloxifene, and Aromatase Inhibitor-resistant Breast Cancer Xenograft Tumors**
Eric A. Ariazi, Heather E. Cunliffe, Amanda L. Willis, Catherine M. Mancini, Yoganand Balagurunathan, Shaun D. Gill, Jennifer R. Pyle, Heather A. Shupp, V. Craig Jordan

*Abstract #B51 was published in the 2007 Proceedings of the American Association for Cancer Research Special Conference in Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications.*
**Long-term estrogen deprivation of breast cancer cells causes significant genomic evolution and development of enhanced malignant behavior.**
Catherine M. Mancini, Joan S. Lewis-Wambi, Eric A. Ariazi, Helen R. Kim, Amanda L. Willis, V. Craig Jordan and Heather Cunliffe

*Abstract #2687 was published in the 2008 Proceedings of the 99th Annual Meeting of the American Association for Cancer Research.*
**Glutathione depletion sensitizes hormone-independent human breast cancer cells to estrogen-induced apoptosis.**
Joan S. Lewis-Wambi, Helen R. Kim, Chris Wambi, V. Craig Jordan

*Abstract #5452 was published in the 2008 Proceedings of the 99th Annual Meeting of the American Association for Cancer Research.*
**GPR30 modulates estrogen-stimulated proliferation of breast and endometrial cancer cells by regulating estrogen receptor alpha homeostasis.**

Eric A. Ariazi, Heather A. Shupp, Jing Peng, Anne L. Donato, Surojeet Sengupta, Catherine G.N. Sharma, Helen R. Kim, Heather E. Cunliffe, Eric R. Prossnitz, V. Craig Jordan.

*Abstract #P2-2 was published in the 2008 Program for the 5th Era of Hope Department of Defense Breast Cancer Research Program Meeting.*
**Glutathione depletion sensitizes hormone-independent human breast cancer cells to estrogen-induced apoptosis.**
Joan S. Lewis-Wambi, Helen R. Kim, Chris Wambi, V. Craig Jordan

*Abstract #P2-17 was published in the 2008 Program for the 5th Era of Hope Department of Defense Breast Cancer Research Program Meeting.*
**Genomic Evolution of Endocrine-Resistant Breast Cancer Cell Lines Reveals Molecular Aberrations Consistent with Biological Phenotype**
Catherine M. Mancini, Coya Tapia, Amanda L. Willis, Joan S. Lewis-Wambi, Eric A. Ariazi, Helen R. Kim, Heather E. Cunliffe, V. Craig Jordan

*Abstract #P2-18 was published in the 2008 Program for the 5th Era of Hope Department of Defense Breast Cancer Research Program Meeting.*
**Comparative Gene Expression Profiling to Identify Unifying and Selective Pathways Involved in Tamoxifen, Raloxifene, and Aromatase Inhibitor-Resistant Breast Cancer Xenograft Tumors**
Eric Ariazi, Heather E. Cunliffe, Michael S. Slifker, Suraj Peri, Amanda L. Willis, Catherine M. Mancini, Heather A. Shupp, Surojeet Sengupta, Jing Peng, Anne L. Donato, Catherine G.N. Sharma, Shaun D. Gill, Jennifer R. Pyle, Karthik Devarajan, Yoganand Balagurunathan, Eric A. Ross, V. Craig Jordan

*Abstract #P2-19 was published in the 2008 Program for the 5th Era of Hope Department of Defense Breast Cancer Research Program Meeting.*
**The Evolution of Drug Resistance to Antihormonal Therapy Exposes A Vulnerability in Breast Cancer**
V. Craig Jordan, Eric A. Ariazi, Joan S. Lewis-Wambi, Ramona F. Swaby, Anton Wellstein, Anna T. Riegel, Heather E. Cunliffe

*Abstract #P2-20 was published in the 2008 Program for the 5th Era of Hope Department of Defense Breast Cancer Research Program Meeting.*
**Single Arm Phase 2 Study of Pharmacologic Dose Estrogen in Postmenopausal Women With Hormone Receptor-Positive Metastatic Breast Cancer After Failure of Sequential Endocrine Therapies**
Ramona Swaby, Mary B. Daly, Nancy E. Davidson, Eric A. Ross, Lori J. Goldstein, V. Craig Jordan

*Abstract #P2-27 was published in the 2008 Program for the 5th Era of Hope Department of Defense Breast Cancer Research Program Meeting.*
**Proteomic Analysis of Phosphotyrosine-Containing Protein Complexes During Estrogen-Induced Proliferation and Apoptosis in MCF-7 Human Breast Cancer Cells**
Anton Wellstein, Benjamin Kagan, Zhang-Zhi Hu, Cathy Wu, Hongzhan Huang, Lihua Zhang, Habtom W. Ressom, Francoise Seillier-Moiseiwitsch, Anna T. Riegel, V. Craig Jordan

## Presentations

**2007**
Jordan, VC.  Oestrogen is bad for patients with breast cancer? Controversies in Breast Cancer, Edinburgh, September 3-4, 2007
Jordan, VC.  What is New in Chemoprevention for Breast Cancer? Aultman Cancer Conference, Ohio, Septmber 14-15, 2007

Jordan, VC. Background, scientific rationale, and organization, Center of Excellence External Advisory Board meeting, September 18, 2007

Jordan, VC. Oestrogen induced breast cancer cell apoptosis, 12th World Congress in Advances in Oncology and 10th International Symposium on Molecular Medicine, Crete, October 11-13, 2007.

Jordan, VC. Thirty-Five Years of Breast Cancer Chemoprevention, Molecular Targets for Cancer Prevention Diagnosis and Treatment, Keynote Lecture, Lemesos, Cyprus, 2007.

Jordan, VC. The consequences of long-term antihormonal therapy: Oestrogen-induced apoptosis, Molecular Targets for Cancer Prevention Diagnosis and Treatment, Lemesos, Cyprus, 2007

Jordan, VC. Hormonal therapy of breast cancer: a move to aromatase inhibitors, 4th Oncology Fall Conference, Hoover, Alabama, October 20-21, 2007

Jordan, VC. Hormones and Women's Cancer, Keystone Program, 2007

Jordan, VC. Practical Issues in Endocrine Chemoprevention, 9th Lynn Sage Meeting, Chicago, September 27-30, 2007

Jordan, VC. Tamoxifen, the first targeted therapy for the treatment of breast cancer, Medical Oncology Fellows Conference, Fox Chase Cancer Center, Philadeplphia, September 24, 2007

Jordan, VC. Current Solutions to Breast Cancer Treatment with Hormonal Therapy, 8ème Biennale Monégasque de Cancérologie, Monte Carlo, Monaco, January 23-28, 2007.

Jordan, VC. Estrogens and anti-estrogens in the life and death of breast cancer cells: new treatment opportunities, Ohio State University Grand Rounds, September 21, 2007

Jordan, VC. Hormonal Treatments: Past, Present, and Future, Living Beyond Breast Cancer Annual Fall Conference, November 17, 2007

**2008**

Jordan, VC. The Science of Selective Estrogen Receptor Modulators and their Clinical Application, AAAS Annual Meeting, Boston, February 14-18, 2008

Jordan, VC. Current Status of Breast Cancer Prevention, 25th Annual Miami Breast Cancer Conference, Orlando, Florida, February 20-23 2008

Jordan, VC. New Insights into the role of estrogen in the life and death of breast cancer cells, Visiting Professor Seminar Speaker Series, University of New Mexico Cancer Center, March 3, 2008

Jordan, VC. Protein-Centric Integration and Functional Analysis of Cancer Omics Data, USHUPO 4th Annual Conference, Bethesda, MD, March 16-19, 2008

Jordan, VC. Tamoxifen and Raloxifene: the First Selective Estrogen Receptor Modulators, 12th Annual Interdisciplinary Women's Health Research Symposium, March 28, 2008

Jordan, VC. Development and current role of tamoxifen and raloxifene in the treatment and prevention of breast cancer and osteoporosis, Institut für Klinische Pharmakologie, Stuttgart, Germany, April 29, 2008

Jordan, VC. Defeating Drug Resistance to SERMs: Building on the Success of Tamoxifen and Raloxifene, Institut für Klinische Pharmakologie, Stuttgart, Germany, May 2008

Jordan, VC. Targeting Oestrogen to Kill Breast Cancer Cells, First International Conference, Forli, Italy, May 16, 2008

Jordan, VC. Translational Research in Breast Cancer at the UWCCC that changed Medical Practice, Department of Medicine Research Day, Madison WI, May 29, 2008

Jordan, VC. The Paradoxical Actions of Estrogen in Breast Cancer: Survival or Death!, 38th Karnofsky Award Lecture, The American Society of Clinical Oncology, May 31, 2008

Jordan, VC. Model Systems of Anti-hormonal Therapy to Evaluate New Targeted Treatments for Breast and Endometrial Cancer, Pfizer, June, 2008

Cunliffe, H. Genomic Evolution of Endocrine-Resistant Breast Cancer Cell Lines Reveals Molecular Aberrations Consistent with Biological Phenotype, Session 8-1, 5th Era of Hope Meeting, Baltimore, MD, June 26, 2008

Ariazi EA. Comparative Gene Expression Profiling to Identify Unifying and Selective Pathways Involved in Tamoxifen, Raloxifene, and Aromatase Inhibitor-Resistant Breast Cancer Xenograft Tumors, Session 8-2, 5th Era of Hope Meeting, Baltimore, MD, June 26, 2008

Jordan, VC. The Evolution of Drug Resistance to Anti-hormonal Therapy Exposes a Vulnerability in Breast Cancer, Session 36-5, 5th Era of Hope Meeting, Baltimore, MD, June 28, 2008

Ariazi EA, Jordan, VC. Estrogen receptors as therapeutic targets in breast cancer, 236th American Chemical Society National Meeting, Division of Computers in Chemistry, Final paper number 27, Philadelphia, PA, August 17-21, 2008

Brailoiu E, Brailoiu GC, Dun SL, Deliu E, Ariazi EA, Arterburn JB, Prossnitz ER, Oprea TI, Jordan VC, Dun NJ. Localization, distribution, and pharmacology of G protein-coupled estrogen receptor GPR30, 236th American Chemical Society National Meeting, Division of Computers in Chemistry, Final paper number 28, Philadelphia, PA, August 17-21, 2008

Jordan, VC. Modulation of Oestrogen Action: By Looking Back We Can See The Way Forward, Controversies Meeting, Edinburgh, September 2, 2008

Jordan, VC. Defeating Drug Resistance to SERMs: Building on the Success of Tamoxifen and Raloxifene, Lund, Sweden, September 4, 2008

Jordan, VC. Oestrogen in the Life and Death of Breast Cancer Cells: The Consequences of Anti-Hormonal Therapy, Nobel Symposium, Svartsjö, Sweden, September 9, 2008

Jordan, VC. Oestrogen-induced apoptotic mechanisms and their potential application in breast cancer therapy, 18th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, Seefeld, Austria, September 20, 2008

Jordan, VC. Breast Cancer Research and Treatment by Blocking Estrogen Action, National Breast Cancer Coalition, Washington D.C, 2008

## Grants

**V. Craig Jordan (Principal Investigator)**
**Eric A. Ariazi (Co-Principal Investigator)**
Submitted: Pre-application to the Susan G. Komen for the Cure 2009 Grants Program
Application Title: The G protein-coupled estrogen receptor GPR30's role in antihormone-sensitive to antihormone-resistant breast cancer.
Mechanism: Investigator Initiated Research
Pre-application Submission Date: 07/01/08

The Pre-application was found responsive to the Request for Applications (RFA), and consequently, invited for a full application.

Full-application Submission Date: 09/04/08

## Awards and Honorary Memberships

**V. Craig Jordan**

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| American Cancer Society Southeast Region Pennsylvania Division Scientific Research Award for Outstanding Accomplishments in Translational Research | October 2007 |
| Beneficiary of the Genuardi's Markets' (a division of Safeway Inc.) campaign to raise funds for breast cancer research | October 2007 |
| Honorary Member of the Royal Pharmaceutical Society of Great Britain | April 2008 |
| Recipient of the 38th David A. Karnofsky Award and Lecture from the American Association for Clinical Oncology | June 2008 |
| Honorary Member of the Royal Society of Medicine, UK | July 2008 |

**Eric A. Ariazi**

Faculty Appointment (secondary appointment) - Assistant Professor, Department              June 2008
  of Pharmacology, Temple University School of Medicine, Philadelphia, PA

## CONCLUSION

Our interconnected network is effectively evaluating the $E_2$ regulated signal transduction pathways for the initiation of growth or apoptosis in breast cancer cells. This is being accomplished by using unique antihormone resistant models developed in our laboratory over the past two decades.  We are progressing on schedule with the systematic generation of samples for proteomic and gene expression microarray studies of cell lines at the FCCC **(Task 2/FCCC).** In year 1, we generated 24 h protein samples, and in year 2, 2 h proteomic samples. We also produced in year 1, RNA samples from MCF-7/WS8 and MCF-7/5C cells for microarray, and during year 2, we produced MCF-7/2A RNA samples. Our extensive quality control system continues to ensure the correct allocation of samples to treatment groups before further proteomic and microarray analyses. Proteomics and pathway studies of the cell line models in which AIB1-interacting and Tyrosine-phosphorylated proteins are being identified by MS are well underway **(Task 3/GU)**. We will soon complement these proteomic studies by identifying proteins which interact with ERα. We have completed an extensive pilot gene expression microarray study of tumor model systems grown in athymic mice **(Task 4/FCCC)**.  Our study *in vivo* is providing excellent data for pathway analysis as indicated by identification of deregulated genes which modulate ERα activities, and identification of Snail1, which drives EMT, and could be involved in $E_2$-induced apoptosis. Additionally, the *in vivo* microarray study identified candidate biomarkers that can be evaluated in the clinical trial specimens, once these specimens are collected. We have developed the methodological approaches to analyze time course microarray data using the MCF-7/WS8 and MCF-7/5C microarray data **(Task 4/TGen)**. The MCF-7/2A cells will by hybridized to microarrays during year 3, and data from all three cell lines will be analyzed together using the methodologies developed during year 2. We have also optimized conditions for HT-siRNA screen **(Task 4/TGen)**. Our unique ability to compare and contrast breast cancer models of $E_2$ regulated cell growth, hormone independent growth, SERM stimulated growth, and $E_2$-induced apoptosis is creating a unique view of $E_2$ regulation in cells not previously appreciated.  We report our investigation of specific proteins that act as a mediator of estrogen action that are critical for $E_2$-dependent growth and which are amplified in estrogen deprived cells.  We report the regulation of the survival signal Bcl-2 that is regulated by XBP-1 **(Task 2/FCCC)**. Additionally, based on gene array analysis of our unique estrogen deprived cell lines, we have identified and now published about the dramatic elevations of the invasion protein, CEACAM6 compared with antihormone treatment naïve breast cancer cells **(Task 2/FCCC)**. These examples illustrate the power of our integrated approach to deciphering the signal transduction pathways in our unique models using genomics and proteomics.  Most importantly, in future years, the developing survival and apoptotic map will be interrogated using tissue samples from our clinical trials using $E_2$ to treat patients who have developed resistance to antihormonal therapy.  We have successfully started to recruit to our clinical trial of 12 weeks of high dose $E_2$ (30 mg daily) therapy for patients who have successfully been treated with and failed at least two successive antihormonal therapies **(Task 1)**.

## APPENDIX

### FCCC

Lewis-Wambi JS, Kim HR, Wambi C, Patel R, Pyle J, Klein-Szanto AJ, Jordan VC.  Buthionine sulfoximine sensitizes hormone-resistant human breast cancer cells to estrogen-induced apoptosis. *Breast Cancer Res 2008* (submitted).

Lewis-Wambi JS, Cunliffe HE, Kim HR, Willis AL, Jordan, VC.  Overexpression of CEACAM6 promotes migration and invasion of oestrogen-deprived breast cancer cells. *Eur J Cancer* 2008: 44(12):1170-79.

## Georgetown

Oh AS, Lahusen JT, Chien CD, Fereshteh MP, Zhang X, Dakshanamurthy S, Xu J, Kagan BL, Wellstein A, Riegel AT.  Tyrosine phosphorylation of the nuclear receptor coactivator AIB1/SRC-3 is enhanced by Abl kinase and is required for its activity in cancer cells. *Mol Cell Biol.* 2008 Sep 2 (Epub ahead of print).

# Exemestane's 17-hydroxylated metabolite exerts biological effects as an androgen

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## Abstract

**Aromatase inhibitors (AI) are being evaluated as long-term adjuvant therapies and chemopreventives in breast cancer. However, there are concerns about bone mineral density loss in an estrogen-free environment. Unlike nonsteroidal AIs, the steroidal AI exemestane may exert beneficial effects on bone through its primary metabolite 17-hydroexemestane. We investigated 17-hydroexemestane and observed it bound estrogen receptor α (ERα) very weakly and androgen receptor (AR) strongly. Next, we evaluated 17-hydroexemestane in MCF-7 and T47D breast cancer cells and attributed dependency of its effects on ER or AR using the antiestrogen fulvestrant or the antiandrogen bicalutamide. 17-Hydroexemestane induced proliferation, stimulated cell cycle progression and regulated transcription at high sub-micromolar and micromolar concentrations through ER in both cell lines, but through AR at low nanomolar concentrations selectively in T47D cells. Responses of each cell type to high and low concentrations of the non-aromatizable synthetic androgen R1881 paralleled those of 17-hydroexemestane. 17-Hydroexemestane down-regulated ERα protein levels at high concentrations in a cell type–specific manner similarly as 17β-estradiol, and increased AR protein accumulation at low concentrations in both cell types similarly as R1881. Computer docking indicated that the 17β-OH group of 17-hydroexemestane relative to the 17-keto group of exemestane contributed significantly more intermolecular interaction energy toward binding AR than ERα. Molecular modeling also indicated that 17-hydroexemestane interacted with ERα and AR through selective recognition motifs employed by 17β-estradiol and R1881, respectively. We conclude that 17-hydroexemestane exerts biological effects as an androgen. These results may have important implications for long-term maintenance of patients with AIs. [Mol Cancer Ther 2007;6(11):2817–27]**

## Introduction

The third-generation aromatase inhibitors (AI) anastrozole (Arimidex; refs. 1, 2), letrozole (Femara; refs. 3, 4), and exemestane (Aromasin; refs. 5, 6), by virtue of blocking extragonadal conversion of androgens to estrogens and giving rise to an estrogen-depleted environment, exhibit improved efficacy over tamoxifen in the adjuvant therapy of estrogen receptor (ER) positive breast cancer in post-menopausal women (7). Clinical trials evaluating these AIs showed a reduced incidence of contralateral primary breast cancer in the AI groups compared with tamoxifen (1 6); hence, AIs are currently being evaluated as chemopreventives in ongoing studies (8). AIs also exhibit reduced overall toxicity compared with tamoxifen (1 6, 9), but the toxicity profiles are different: tamoxifen is associated with increased incidences of thromboembolic events and endometrial cancer, whereas AIs are associated with decreased bone mineral density (BMD), coupled with an increased risk of bone fractures (10 12) and severe musculoskeletal pain that limits patient compliance (13, 14). Because the available third-generation AIs all exhibit similar efficacies, the selection of a specific AI for long-term adjuvant therapy of breast cancer and as a chemopreventive in healthy women at high risk for breast cancer will likely be determined by safety and tolerability profiles.

AIs fall into two classes, steroidal as represented by exemestane, which acts as a suicide inhibitor of aromatase, and nonsteroidal including anastrozole and letrozole, which reversibly block aromatase activity (7). Possibly due to its steroid structure, exemestane may exhibit a unique pharmacology distinct from the nonsteroidal AIs. In two preclinical studies by Goss et al. (15, 16), exemestane was given to female ovariectomized rats, an animal model

of osteoporosis, and found to reduce bone resorption markers and increase BMD and bone strength, whereas lowering serum cholesterol and low-density lipoprotein levels compared with ovariectomized controls. One of these preclinical studies also evaluated the nonsteroidal AI letrozole, but in contrast, found no benefit of letrozole on bone or lipid profiles (16). In a clinical study investigating the effects of 2 years of exemestane on bone compared with placebo without prior tamoxifen therapy in patients with surgically resected breast cancer at low risk for recurrence, exemestane did not enhance BMD loss in lumbar spine and only modestly enhanced BMD loss in the femoral neck compared with the placebo group (17). Interestingly, in this study, exemestane promoted bone metabolism by increasing levels of both bone resorption and formation markers (17). However, a clear-cut advantage of exemestane versus the nonsteroidal AIs on bone safety has not been shown in humans, possibly because all other clinical studies compared the AI to tamoxifen (9, 12, 18) or the AI to placebo with prior tamoxifen therapy (10, 11). Drawing conclusions from these studies is difficult because tamoxifen preserves BMD, thereby protecting against fractures, and withdrawal of tamoxifen may have lasting effects on BMD (19).

Maintenance of BMD in women is a known estrogenic effect (20). However, androgen receptors (AR) are also expressed in multiple bone cell types (21, 22), and studies show that androgens maintain BMD in ovariectomized rats (23, 24) and in women (21, 25–27). In ovariectomized rats, physiologic concentrations of androstenedione, a weak androgen and a substrate of aromatase, reduced loss of bone, and the antiandrogen bicalutamide abrogated this effect (23), but anastrozole did not (23). Therefore, the protective effect of androstenedione on maintenance of BMD was androgen mediated and not due to aromatization of androstenedione to estrogen. Furthermore, the non-aromatizable androgen 5α-dihydrotestosterone has been shown to stimulate bone growth in osteopenic ovariectomized rats (24). In pre- and postmenopausal women, endogenous androgen levels correlate with BMD (25, 26). Furthermore, a study comparing estrogen to a synthetic androgen in postmenopausal osteoporotic women showed that both steroids were equally effective in reducing bone resorption (27). Also, a 2-year double-blind trial showed that estrogen plus a non-aromatizable androgen significantly improved BMD over estrogen alone in surgically menopausal women (28). Therefore, exogenous androgens promote BMD maintenance in women when used alone (27) and in conjunction with estrogen (28).

Although exemestane does not bind ER, it is structurally related to androstenedione and has weak affinity for AR (29, 30). At high doses, exemestane exerts possible androgenic activity *in vivo* by inducing an increase in ventral prostate weight in immature castrated rats (29). Recently, Miki et al. (22) showed in human osteoblast hFOB and osteosarcoma Saos-2 cells that exemestane promoted proliferation, which was partially blocked by the anti-androgen hydroxyflutamide, and increased alkaline phosphatase activity. However, metabolites of exemestane may

be mediating these effects. Exemestane is given p.o. at 25 mg/day and rapidly absorbed, showing peak plasma levels within 2 to 4 h and a direct relationship between dosage and peak plasma levels after single (10–200 mg) or repeated doses (0.5–50 mg; refs. 30, 31). Single-dose studies suggested that exemestane has a short elimination half-life, but multiple-dose studies show its terminal half-life to be about 24 h. Exemestane undergoes complex metabolism, and the primary metabolite in plasma has been identified as 17-hydroexemestane, which accumulates to a concentration of about 10% of its parent compound (30). Taking the possible action of metabolites into consideration, Goss et al. (16) administered 17-hydroexemestane to ovariectomized rats and found that it produced the same bone-sparing effects and favorable changes in circulating lipid levels as exemestane. Also, Miki et al. (22) stated that 17-hydroexemestane promoted proliferation of the osteoblast and osteosarcoma cells similar to exemestane, but the data were not shown, and the authors did not further explore 17-hydroexemestane activities. Additionally, Miki et al. (22) showed that the osteoblasts efficiently metabolized androstenedione to testosterone, which involves the reduction of the 17-keto group of androstenedione to a hydroxyl group. Similar metabolism would convert exemestane to 17-hydroexemestane, and thus, activities of exemestane in the osteoblasts may have been mediated by a metabolite of exemestane. Hence, a thorough investigation of exemestane and 17-hydroexemestane activities through ER and AR is warranted to provide evidence regarding whether exemestane could display a more favorable safety and toxicity profile than nonsteroidal AIs for long-term adjuvant use and as a chemopreventive of breast cancer in postmenopausal women. Therefore, we evaluated the pharmacologic actions of exemestane and its primary metabolite 17-hydroexemestane on ER- and AR-regulated activities in a range of cellular and molecular assays. First, we determined the relative binding affinity (RBA) of 17-hydroexemestane to ERα and AR. Next, using MCF-7 and T47D breast cancer cells, we examined the ability of 17-hydroexemestane to stimulate cell proliferation and cell cycle progression (Supplementary Material)[4] via ER and AR, to regulate ER- and AR-dependent transcription, and to modulate ERα and AR protein levels. Lastly, we investigated intermolecular interactions between 17-hydroexemestane and ERα and AR using molecular modeling.

## Materials and Methods
### Compounds and Cell Lines
Exemestane and 17-hydroexemestane were provided by Pfizer. Fulvestrant (ICI 182,780, Faslodex) and bicalutamide (Casodex) were provided by Dr. Alan E. Wakeling and Dr. Barrington J.A. Furr (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom), respectively. All other

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[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

compounds were obtained from Sigma-Aldrich, and cell culture reagents were from Invitrogen. All test agents were dissolved in ethanol and added to the medium at 1:1,000 (v/v). MCF-7/WS8 and T47D:A18 human mammary carcinoma cells, clonally selected from their parental counterparts for sensitivity to growth stimulation by $E_2$ (32), were used in all experiments indicating MCF-7 and T47D cells. Cells were maintained in steroid-replete RPMI 1640, but 3 days before all experiments, were cultured in steroid-free media as previously described (32, 33).

### Competitive Hormone-Binding Assays

Competitive hormone-binding assays were conducted using fluorescence polarization based ERα and AR Competitor Assay kits (Invitrogen) as previously described (34).

### Cellular Proliferation Assays

Cellular proliferation following 7 days in culture was determined by DNA mass per well in 12-well plates using the fluorescent DNA dye Hoechst 33258 as previously described (32).

### Reporter Gene Assays

Reporter gene assays were conducted by transfecting cells with either an ERE(5x)-regulated (pERE(5x)TA-ffLuc; ref. 33) or ARE(5x)-regulated (pAR-Luc; Panomics) firefly luciferase expression plasmid and co-transfected with a basal TATA promoter-regulated (pTA-srLuc) *Renilla* luciferase expression plasmid as previously described (33).

### Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was used to determine AR and ribosomal large phosphoprotein subunit P0 (RLP0; 36B4) mRNA levels as previously described (35).

### Immunoblot Analyses

Immunoblots, prepared as previously described (33), were probed with primary antibodies against AR (AR 441; Lab Vision), ERα (AER 611; Lab Vision), and β-actin (AC-15; Sigma-Aldrich).

### Molecular Modeling and Virtual Docking Calculations

The three-dimensional conformations for $E_2$, 17-hydroexemestane, exemestane, R1881, and dexamethasone were generated with Omega version 2.1 software (OpenEye Scientific Software). These compounds were docked using the following X-ray crystallographic structures: 1GWR (ERα co-complexed with $E_2$, 2.4-Å resolution; ref. 36) and 1XQ3 (AR co-complexed with R1881, 2.25-Å resolution; ref. 37). ERα and AR ligand-binding pockets were built using a ligand-centered box and the receptor-bound conformation of the respective ligand: $E_2$ (for 1GWR) and R1881 (for 1XQ3). The volume of the cavity differs for the two receptors: 648 $Å^3$ for 1GWR and 532 $Å^3$ for 1XQ3. All receptor and ligand bonds were kept rigid. The receptor structures were filled with water because ERα (38) and AR crystal structures (39) indicate that specific stable hydrogen bond (H-bond) networks form among particular water molecules, ligands, and amino acid side chains. Docking was done with FRED version 2.2 software (OpenEye) using a short refinement step for the ligands within the receptor and using the MMFF94 force field. The best 30 conformations for each compound were compared and ranked by FRED's Chemscore function. For each ligand-

docked receptor evaluated, the docked conformation with the lowest total intermolecular interaction energy (kJ/mol) was selected. To address whether water could be displaced by a compound during the process of binding, docking calculations were also done using receptors modeled with water removed as presented in Supplementary Table S1[4] and the differences between the methods in Supplementary Table S2.[4]

### Curve Fitting and Statistical Analyses

All statistical tests, curve fitting, and determination of half-maximal inhibitory concentrations ($IC_{50}$) and half-maximal effective concentrations ($EC_{50}$) were done using GraphPad Prism 4.03 (GraphPad Software). Significant differences were determined using one-way ANOVA with Bonferroni multiple comparison post-test.

## Results

### Experimentally Determined Binding of 17-Hydroexemestane and Exemestane to ERα and AR

Structures of the compounds relevant to these studies, the steroidal AI parent compound exemestane, its primary metabolite 17-hydroexemestane, $E_2$, and the synthetic non-aromatizable androgen R1881, are shown in Fig. 1A. Importantly, the only difference between parental exemestane and its metabolite 17-hydroexemestane is a hydroxyl group in the metabolite in place of a ketone in the parent compound at the 17β position, whereas both compounds share a 3-keto group. For steroidal estrogens, elimination or modification of the 17β-OH group reduces binding to ERα, but that of the 3-OH group is much more dramatic (40). For steroidal androgens, the trend is reversed; elimination or modification of the 17β-OH group is more significant for AR binding than that of the 3-keto group (41). The 3-keto group found in both exemestane and 17-hydroexemestane also favors binding to AR (41).

We tested the binding of exemestane and 17-hydroexemestane to ERα and AR using fluorescence polarization based competitive hormone-binding assays (Fig. 1B and C; Table 1). For purposes of comparison, compound affinities were arbitrarily categorized with respect to their RBAs as strong (100 to ≥1), moderate (<1 to ≥0.1), weak (<0.1 to ≥0.01), very weak (<0.01 to detectable binding defined as 50% competition), and inactive (compound did not compete for at least 50% binding). $E_2$ competitively bound ERα with an $IC_{50}$ of $1.33 \times 10^{-9}$ mol/L (RBA = 100; Fig. 1B), and R1881 competitively bound AR with an $IC_{50}$ of $1.34 \times 10^{-8}$ mol/L (RBA = 100; Fig. 1C). Considering ERα (Fig. 1B), both R1881 and 17-hydroexemestane competed for binding to ERα with $IC_{50}$s of $1.02 \times 10^{-6}$ mol/L (RBA = 0.130) and $2.12 \times 10^{-5}$ mol/L (RBA = 0.006), respectively, which categorized R1881 as a moderate and 17-hydroexemestane as a very weak ERα ligand. Neither exemestane nor dexamethasone significantly competed for binding to ERα. Regarding AR (Fig. 1C), 17-hydroexemestane and exemestane competed for binding to AR with $IC_{50}$s of $3.96 \times 10^{-8}$ mol/L (RBA = 33.8) and $2.03 \times 10^{-6}$ mol/L (RBA = 0.658), respectively, which classified
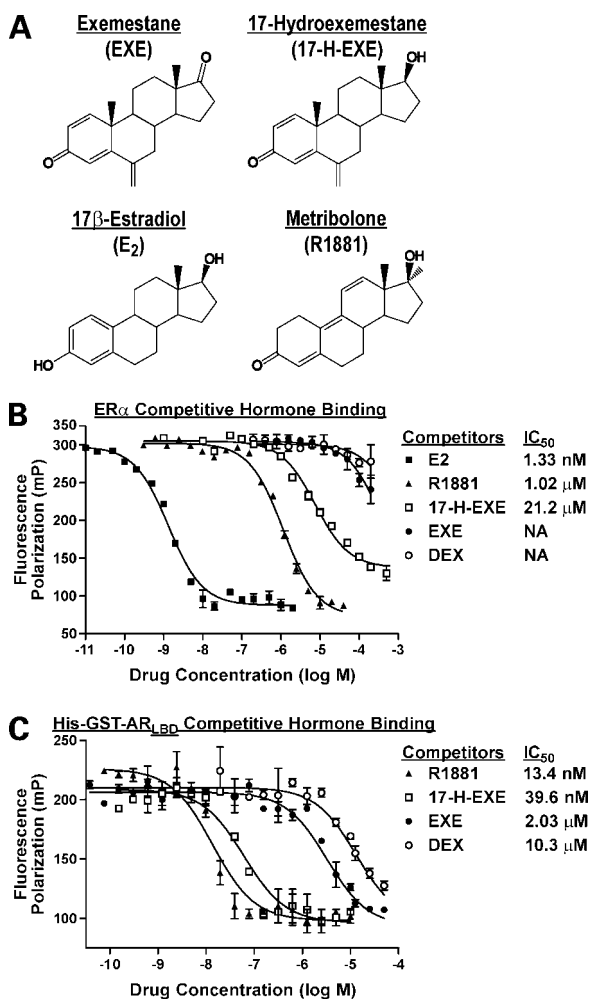
**Figure 1.** Compounds examined in this study and their RBAs for ERα and AR. **A,** structures of exemestane, its primary metabolite 17 hydroexemestane $E_2$, and R1881. ERα (**B**) and AR (**C**) fluorescence polarization based competitive hormone binding assays. Baculovirus pro duced human ERα and rat AR ligand binding domain tagged with a His glutathione *S* transferase epitope (His GST $AR_{LBD}$) were used at final concentrations of 15 and 25 nmol/L, respectively. The fluorescently labeled ERα and AR ligands, Fluormone ES2 and Fluormone AL Green, respectively, were both used at a final concentration of 1 nmol/L. The competing test compounds were $E_2$, R1881, 17 hydroexemestane, exemestane, and dexamethasone (DEX) as indicated. *Point,* mean of triplicate determinations; *bars,* 95% confidence intervals. Curve fitting was done using GraphPad Prism software (version 4.03). $IC_{50}$s corresponding to a half maximal shift in polarization values of the test compounds were determined using the maximum and minimum polariza tion values of the $E_2$ competitive binding curve for ERα or of the R1881 competitive binding curve for AR as appropriate.

17-hydroexemestane as a strong and exemestane as a weak AR ligand. However, dexamethasone would also be categorized as a weak AR ligand. Hence, the observed very weak ERα binding and strong AR binding of 17-hydroexemestane was consistent with what previously reported structure-activity relationships (40, 41) would have predicted due to reduction of the 17-keto group in exemestane to a 17β-OH in the metabolite.

## Proliferation Responses to 17-Hydroexemestane and Exemestane

We examined the effects of exemestane and 17-hydro-exemestane on 7 days of proliferation in ERα- and AR-positive MCF-7 and T47D mammary carcinoma cells (Fig. 2). As expected, both cell lines were growth stimulated by $E_2$, with growth $EC_{50}$s of $1.7 \times 10^{-12}$ mol/L $E_2$ for MCF-7 cells (Fig. 2A) and $7.1 \times 10^{-12}$ mol/L $E_2$ for T47D cells (Fig. 2B). These growth responses to $E_2$ were completely blocked by fulvestrant (all *P* values <0.001), validating the $E_2$ responsiveness via ER in these cell lines.

Both cell lines were also growth stimulated by R1881 (Fig. 2A and B) and 17-hydroexemestane (Fig. 2C and D), whereas exemestane did not exert any significant effect on proliferation (Fig. 2C and D). Considering MCF-7 cells, R1881 exhibited a growth $EC_{50}$ of $2.4 \times 10^{-8}$ mol/L (Fig. 2A), or approximately 4 orders of magnitude higher than that of $E_2$. Similarly, 17-hydroexemestane exhibited a growth $EC_{50}$ of $2.7 \times 10^{-6}$ mol/L in MCF-7 cells (Fig. 2C) or approximately 6 orders of magnitude higher than that of $E_2$. These growth responses to R1881 and 17-hydro-exemestane in MCF-7 cells were completely blocked by cotreatment with fulvestrant (Fig. 2A and B; both *P* values <0.001). Therefore, whereas R1881, a non-aromatizable synthetic androgen, stimulated growth of MCF-7 cells, it did so by acting through ER. Hence, at high concentrations, R1881 exerted estrogenic activity. Similarly, at high concentrations, 17-hydroexemestane also exerted estrogen-ic activity and stimulated growth of MCF-7 cells by acting through ER.

Interestingly, in T47D cells, the growth response to R1881 and 17-hydroexemestane followed an apparent bimodal pattern, which was different than in MCF-7 cells. In T47D cells, proliferative effects of high concentrations of R1881 ($5 \times 10^{-6}$ mol/L; Fig. 2B) and 17-hydroexemestane ($5 \times 10^{-6}$ mol/L; Fig. 2D) were only partially blocked by fulvestrant (both *P* values <0.001), down to the level of growth observed at nanomolar concentrations of these compounds. However, proliferative effects of lower con-centrations of R1881 ($10^{-9}$ mol/L) and 17-hydroexemestane ($10^{-8}$ mol/L) were completely blocked by the anti-androgen bicalutamide (both *P* values <0.001). Based on these observed levels of inhibition by bicalutamide and fulvestrant, maximal concentrations at which R1881 and 17-hydroexemestane stimulated growth through AR-dependent activities were $10^{-7}$ and $10^{-6}$ mol/L, respectively, and above these concentrations, R1881 and 17-hydro-exemestane stimulated growth through ER-dependent activities. Using this information to define concentration ranges in which these compounds exert AR-mediated or ER-mediated effects in T47D cells, the growth $EC_{50}$s via AR of R1881 and 17-hydroexemestane were $1.0 \times 10^{-10}$ mol/L (Fig. 2B) and $4.3 \times 10^{-10}$ mol/L (Fig. 2D), respectively. Similarly, the growth $EC_{50}$s via ER of R1881 and 17-hydroexemestane in T47D cells were $3.1 \times 10^{-7}$ mol/L (Fig. 2B) and $1.5 \times 10^{-6}$ mol/L (Fig. 2D), respectively. Hence, in T47D cells, both R1881 and 17-hydroexemestane stimulated growth via AR at lower

concentrations and via ER at higher concentrations. These results were consistent with the observed binding affinities of these compounds to ER$\alpha$ (Fig. 1B) and AR (Fig. 1C).

### Cell Cycle Progression Responses to 17-Hydroexemestane

As shown in Supplementary Fig. S1,[4] 17-hydroexemestane at $10^{-8}$ mol/L acted through AR to stimulate S-phase entry in T47D cells by 1.9-fold ($P < 0.001$) but, at $5 \times 10^{-6}$ mol/L, acted through ER to stimulate S-phase entry in MCF-7 cells by 2.2-fold ($P < 0.001$). Hence, 17-hydroexemestane effects on cell cycle progression were consistent with its effects on proliferation (Fig. 2).

### Regulation of ER$\alpha$ and AR Transcriptional Activities by 17-Hydroexemestane

Next, we investigated the ability of 17-hydroexemestane to regulate ER and AR transcriptional activity by transfecting cells with an ERE(5x)-regulated or ARE(5x)-regulated dual-luciferase plasmid set, treating cells with test compounds, and measuring dual-luciferase activity 44 h after treatment (Fig. 3A C). $E_2$ at $10^{-10}$ mol/L induced ERE(5x)-regulated transcription by 19.4-fold in MCF-7 cells (Fig. 3A; $P < 0.001$), and 11.3-fold in T47D cells (Fig. 3B; $P < 0.001$) compared with control-treated cells; this $E_2$-induced transcriptional activity was blocked by fulvestrant (both $P$ values <0.001), validating dependence on ER for ERE(5x)-regulated transcription. At high sub-micromolar and micromolar concentrations, R1881 stimulated ERE(5x)-regulated transcription in both cell lines, with maximal inductions of 22.7-fold at $5 \times 10^{-6}$ mol/L in MCF-7 cells (Fig. 3A; $P < 0.001$), and 7.9-fold at $5 \times 10^{-6}$ mol/L in T47D cells (Fig. 3B; $P < 0.001$) compared with control-treated cells. The ability of R1881 at $5 \times 10^{-6}$ mol/L to induce ERE(5x)-regulated transcription was blocked by fulvestrant (Fig. 3A and B; both $P$ values <0.001), indicating that at high concentrations, R1881 acted as an estrogen. In a similar manner as R1881, 17-hydroexemestane stimulated ERE(5x)-regulated transcription in a concentration-dependent manner at sub-micromolar and micromolar concentrations

(Fig. 3A and B). At $5 \times 10^{-6}$ mol/L, 17-hydroexemestane maximally induced ERE(5x)-regulated transcription by 7.7-fold in MCF-7 cells (Fig. 3A; $P < 0.001$) and 3.3-fold in T47D cells (Fig. 3B; $P < 0.001$) compared with control-treated cells; this transcriptional activation was blocked by fulvestrant (both $P$ values <0.001). Therefore, at high concentrations, 17-hydroexemestane acted as an estrogen and induced ER transcriptional activity.

In a similar manner, AR-dependent transcriptional activity was investigated. T47D cells showed a concentration-dependent induction of ARE(5x)-regulated transcription in response to R1881, with $10^{-9}$ mol/L R1881 inducing transcription by 8.5-fold and $10^{-6}$ mol/L R1881 maximally inducing transcription by 12.7-fold relative to control-treated cells (Fig. 3C; both $P$ values <0.001). Bicalutamide blocked $10^{-9}$ mol/L R1881-mediated induction of ARE(5x)-regulated transcription (Fig. 3C; $P < 0.001$), confirming dependence on AR. MCF-7 cells failed to respond to $10^{-6}$ mol/L R1881 with induction of ARE(5x)-regulated transcription (data not shown), although these cells express AR protein. This supports our prior results that T47D cells were growth stimulated by R1881 through an AR-dependent mechanism (Fig. 2B), but that MCF-7 cells were not (Fig. 2A). As expected, $10^{-6}$ mol/L $E_2$ failed to induce ARE(5x)-regulated transcription (Fig. 3C). Next, 17-hydroexemestane was evaluated in T47D cells and, in a concentration-dependent manner, induced ARE(5x)-regulated transcription with maximal induction of 4.7-fold occurring at $5 \times 10^{-6}$ mol/L relative to control treatment (Fig. 3C; $P < 0.001$). However, because high concentrations of 17-hydroexemestane were needed to induce this synthetic ARE(5x)-regulated promoter, we tested whether lower concentrations of 17-hydroexemestane could modulate endogenous AR mRNA expression, which is known to be negatively feedback regulated by its gene product (42). Using real-time PCR, AR mRNA levels were determined in T47D cells following 24 h of treatment with test compounds (Fig. 3D). R1881 at $10^{-9}$ mol/L significantly down-regulated

**Table 1.** Compound affinity for ER$\alpha$ and AR determined experimentally using a competitive hormone binding assay (Fig. 1B and C), and by computer docking in which receptors were modeled as filled with water

| Compound | Receptor | Competitive hormone binding | | | Intermolecular interaction energy (kJ/mol) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | IC$_{50}$ (mol/L) | 95% CI (mol/L) | RBA (%) | Total score | Lipophilic | H bond | Steric clash | RTB penalty |
| $E_2$ | ER$\alpha$ | $1.33 \times 10^{-9}$ | 1.18  $1.49 \times 10^{-9}$ | 100 | −31.90 | −25.96 | −6.00 | 0.06 | 0 |
| R1881 | ER$\alpha$ | $1.02 \times 10^{-6}$ | 0.90  $1.15 \times 10^{-6}$ | 0.130 | −29.96 | −26.01 | −4.32 | 0.37 | 0 |
| 17 Hydroexemestane | ER$\alpha$ | $2.12 \times 10^{-5}$ | 1.73  $2.61 \times 10^{-5}$ | 0.006 | −29.14 | −27.73 | −3.34 | 1.93 | 0 |
| Exemestane | ER$\alpha$ | NA | | | −27.33 | −25.98 | −3.34 | 1.99 | 0 |
| Dexamethasone | ER$\alpha$ | NA | | | −23.71 | −29.70 | −4.18 | 9.07 | 1.10 |
| R1881 | AR | $1.34 \times 10^{-8}$ | 1.00  $1.79 \times 10^{-8}$ | 100 | −32.75 | −28.47 | −4.56 | 0.28 | 0 |
| 17 Hydroexemestane | AR | $3.96 \times 10^{-8}$ | 2.74  $5.71 \times 10^{-8}$ | 33.8 | −31.95 | −30.54 | −4.76 | 3.35 | 0 |
| Exemestane | AR | $2.03 \times 10^{-6}$ | 1.39  $2.97 \times 10^{-6}$ | 0.658 | −26.48 | −28.80 | −2.11 | 4.43 | 0 |
| Dexamethasone | AR | $1.03 \times 10^{-5}$ | 0.75  $1.43 \times 10^{-5}$ | 0.130 | −24.53 | −32.21 | −2.49 | 9.07 | 1.10 |

Abbreviations: RTB Penalty, rotable bond penalty; NA, not applicable; test compound did not compete for at least 50% binding of ER$\alpha$.
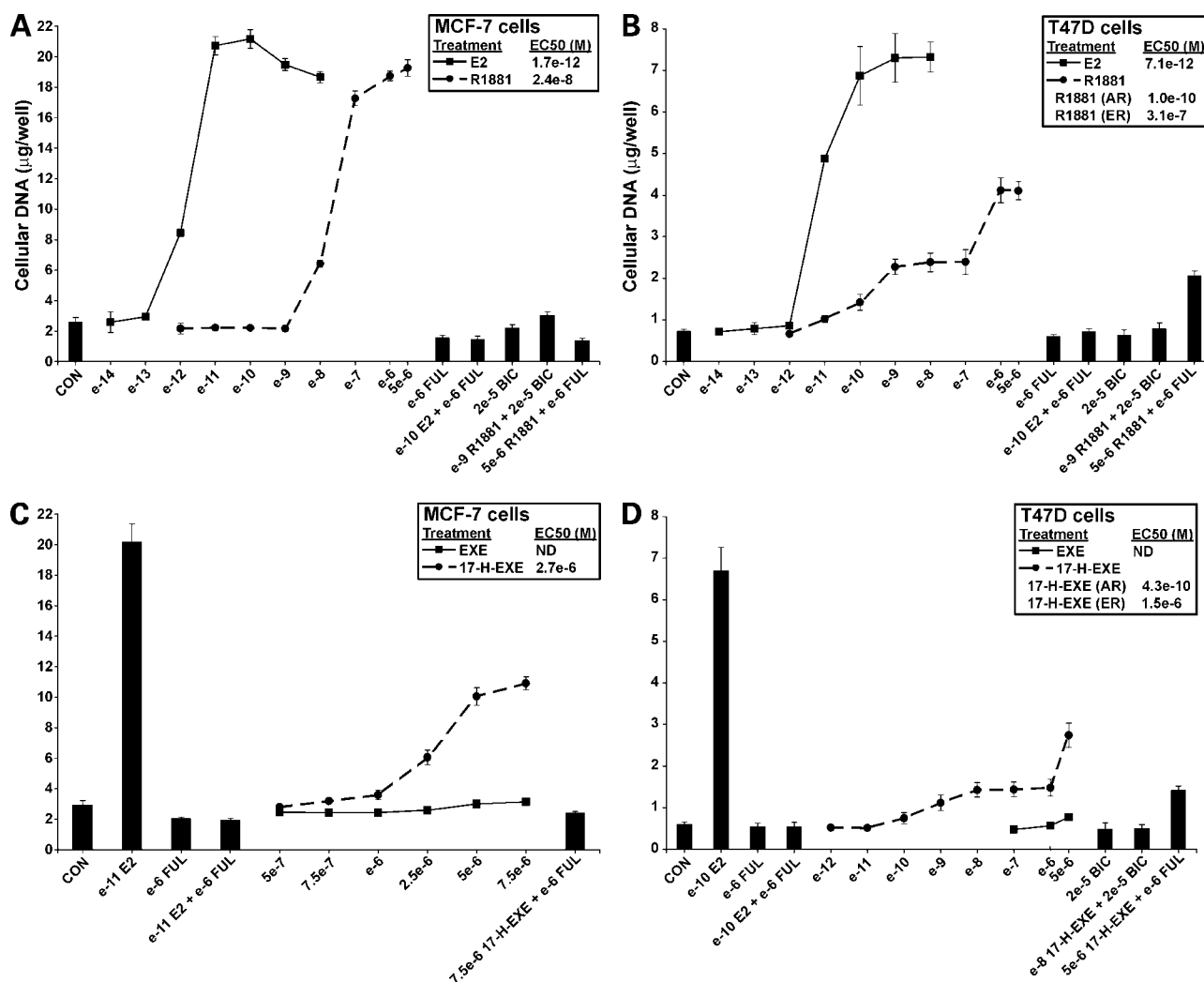
**Figure 2.** 17 Hydroexemestane and R1881 stimulate cellular proliferation. DNA based cellular proliferation assays of (**A**) MCF 7 cells treated with $E_2$ and R1881, (**B**) T47D cells treated with $E_2$ and R1881, (**C**) MCF 7 cells treated with exemestane and 17 hydroexemestane, and (**D**) T47D cells treated with exemestane and 17 hydroexemestane. Cells were cultured in steroid free medium for 3 d before the assays. MCF 7 cells were seeded at 15,000 cells per well and T47D cells at 20,000 cells per well in 12 well plates. Cells were treated on days 0 (the day after seeding), 3, and 6, and then collected on day 7. Cellular DNA quantities were determined using the fluorescent DNA binding dye Hoechst 33258 and compared against a standard curve. Data shown represent the mean of four replicates and SDs. DNA values were fitted to a sigmoidal dose response curve and growth $EC_{50}$s calculated using GraphPad Prism 4.03 software. At high concentrations, 17 hydroexemestane and R1881 increased growth via ER in both cell lines but, at low concentrations, stimulated growth via AR selectively in T47D cells. Abbreviations: CON, control; FUL, fulvetsrant; BIC, bicalutamide.

AR mRNA expression by 48% ($P < 0.001$), whereas $10^{-9}$ mol/L $E_2$ did not (Fig. 3D). Bicalutamide prevented R1881-mediated decrease in AR mRNA expression (Fig. 3D), validating that AR mRNA levels were negatively feedback regulated. Similarly, a low $10^{-8}$ mol/L concentration of 17-hydroexemestane led to a 41% decrease in AR mRNA levels ($P < 0.01$), with increased 17-hydroexemestane concentrations further decreasing AR mRNA expression (Fig. 3D). Bicalutamide blocked 17-hydroexemestane mediated down-regulation of AR mRNA expression ($P < 0.01$), whereas fulvestrant did not (Fig. 3D). Therefore, 17-hydroexemestane acted as an androgen via AR to feedback-regulate the expression of endogenous AR mRNA in T47D cells.

## Modulation of AR and ERα Protein Levels by 17-Hydroexemestane

Androgens and estrogens modulate protein expression levels of their cognate receptors. R1881 stabilizes AR protein allowing its accumulation (43), whereas $E_2$ promotes ERα degradation in a cell type dependent manner (32). Therefore, we investigated the effects of 17-hydroexemestane on AR and ERα protein levels by treating cells with test compounds for 24 h and analyzing receptor levels by immunoblotting. $E_2$ decreased ERα protein levels in MCF-7 (Fig. 4A), but not T47D cells (Fig. 4B), as we have previously shown (32). As expected, fulvestrant promoted ERα protein degradation in both cell lines. $E_2$ did not significantly affect AR protein accumulation in MCF-7 cells

(Fig. 4A), but did down-regulate AR protein levels in T47D cells (Fig. 4B). Also, fulvestrant and $E_2$ plus fulvestrant treatments did not significantly affect AR protein levels in MCF-7 cells (Fig. 4A), but did modestly up-regulate AR protein levels in T47D cells (Fig. 4B). As expected, R1881 caused an increase in accumulation of AR protein in both cell lines (Fig. 4A and B), likely by stabilizing the protein (43). Next, we characterized the effects of low $10^{-8}$ mol/L and high $5 \times 10^{-6}$ mol/L concentrations of 17-hydro-exemestane on ER$\alpha$ and AR expression. The high $5 \times 10^{-6}$ mol/L concentration of 17-hydroexemestane led to de-creased ER$\alpha$ protein levels in MCF-7 (Fig. 4A), but not in T47D cells (Fig. 4B); this pattern indicates that $5 \times 10^{-6}$ mol/L

17-hydroexemestane acted as an estrogen to regulate ER$\alpha$ protein in a cell type dependent manner. Similar to R1881, treatment with low $10^{-8}$ mol/L or high $5 \times 10^{-6}$ mol/L concentrations of 17-hydroexemestane led to increased AR protein accumulation in both cell lines (Fig. 4A and B), indicating that 17-hydroexemestane acted as an androgen likely by stabilizing AR protein. Therefore, 17-hydroexemes-tane modulated ER$\alpha$ and AR protein accumulation as would an estrogen and an androgen, respectively.

## Molecular Docking of 17-Hydroexemestane and Exemestane to ER$\alpha$ and AR

To investigate the mechanism by which 17-hydroexe-mestane binds ER$\alpha$ as a very weak ligand and AR as a
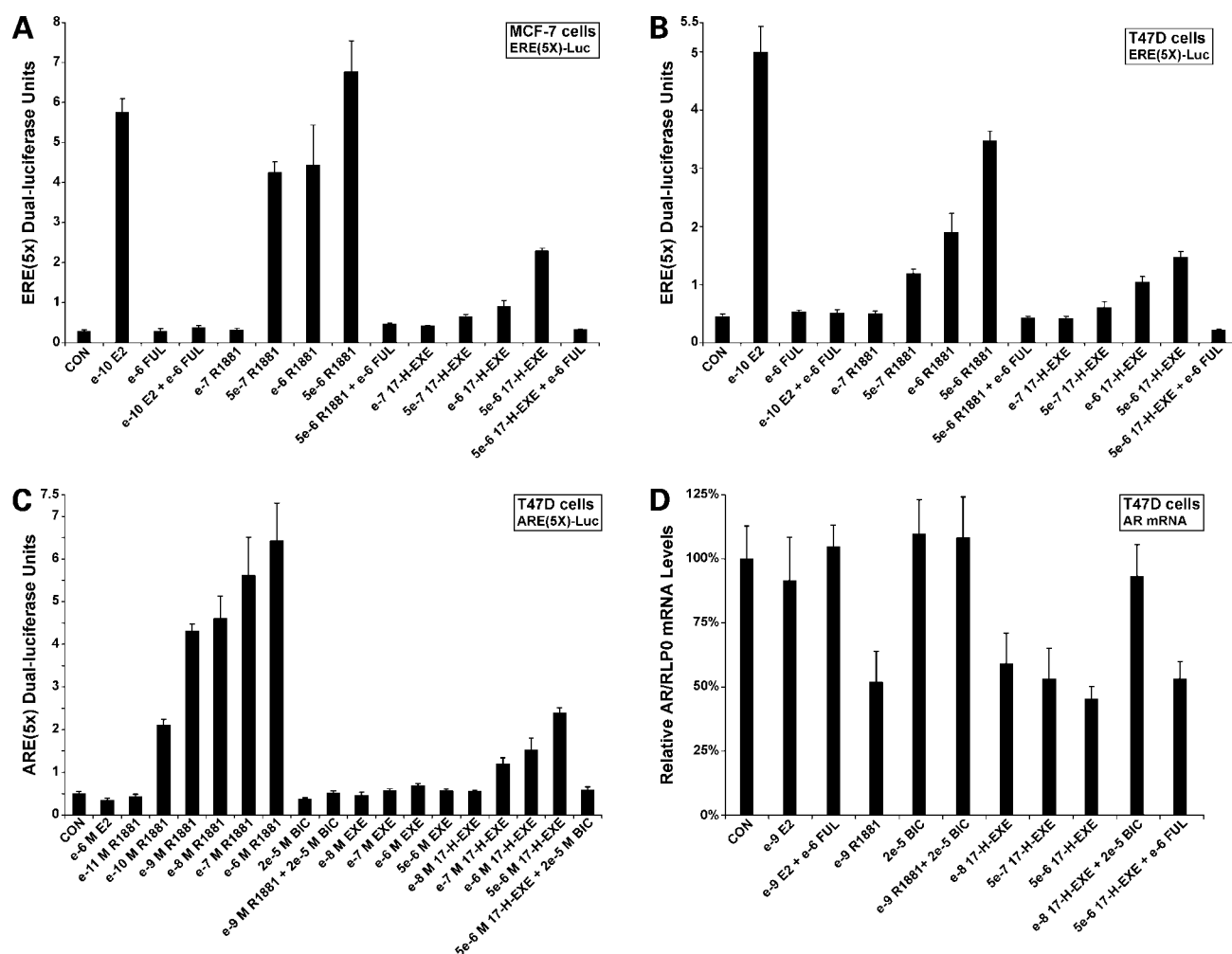


Figure 3. 17 Hydroexemestane and R1881 regulate ER transcriptional activity at high concentrations and AR transcriptional activity at low concentrations. ERE(5x) regulated dual luciferase activity in (**A**) MCF 7 cells and (**B**) T47D cells. (**C**) ARE(5x) regulated reporter gene activity in T47D cells. *A C*, Under steroid free conditions, cells were transiently transfected with pERE(5x)TA ffLuc or pARE(5x) Luc (firefly luciferase reporter plasmids) and the internal normalization control pTA srLuc (*Renilla* luciferase reporter plasmid). Four hours after transfection, cells were treated as indicated and then again the following day. Cells were assayed 44 h after transfection for dual luciferase activity. Data shown are the mean of triplicate determinations and associated SDs. 17 Hydroexemestane and R881 stimulated ERE(5x) regulated transcription in MCF 7 and T47D cells and ARE(5x) regulated transcriptional activity in T47D cells. **D,** AR mRNA levels in T47D cells as determined by real time PCR. T47D cells were treated as indicated for 24 h. RNA was isolated and converted to cDNA. Continuous accumulation of PCR products was monitored using the double strand specific DNA dye SYBR Green. Quantitative measurements of AR mRNA and the endogenous normalization control RLP0 mRNA were determined by comparison to a standard curve of known quantities of serially diluted AR or RLP0 PCR product. The data represent the mean and SDs of three independent samples, each of which was measured in triplicate. 17 Hydroexemestane and R881 down regulated AR mRNA levels at nanomolar concentrations in an AR dependent manner.
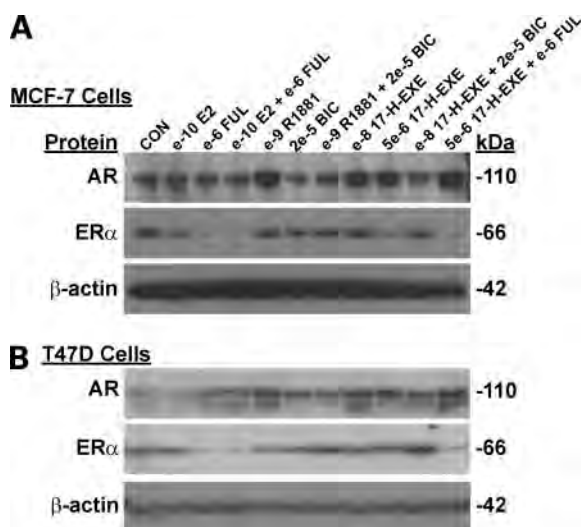
**Figure 4.** 17 Hydroexemestane modulates AR and ERα protein levels. Immunoblot analysis of AR and ERα in (**A**) MCF 7 cells and (**B**) T47D cells. Cells were treated as indicated for 24 h, and 20 μg of cellular protein were resolved by 4% to 12% SDS PAGE and then transferred to a nylon membrane. Membranes were probed for AR, ERα, and β actin, and immunoreactive bands were visualized by chemiluminescence and autoradiography. Cropped blots are shown. 17 hydroexemestane up regulated AR protein levels at $10^{-8}$ mol/L in both cell lines and down regulated ERα in MCF 7 cells at $5 \times 10^{-6}$ mol/L.

strong ligand, molecular models were constructed *in silico*. The trends in the computed intermolecular interaction energies matched the experimentally determined RBAs (Table 1). Superimposition of the docked and crystallographic structures of $E_2$ complexed with ERα (Fig. 5A) and of R1881 complexed with AR (Fig. 5B) showed that the docking models recapitulated the molecular recognition patterns of the crystal structures.

Considering ERα, the intermolecular interaction energies of R1881 and 17-hydroexemestane were less favorable than $E_2$ by 1.94 and 2.76 kJ/mol, respectively, due to decreased H-bond interactions and increased steric clash (Table 1). Exemestane was much less favorable than $E_2$ by 4.57 kJ/mol (Table 1). Hence, the 17β-OH group of 17-hydroexemestane compared with the 17-keto group of exemestane contributed −1.81 kJ/mol toward increased affinity for ERα. Interestingly, the docking calculations suggested that the higher affinity of 17-hydroexemestane over exemestane for ERα was not due to increased H-bonding mediated by the 17β-OH group, but rather increased lipophilic interactions (Table 1) due to a slight repositioning of the compound as a consequence of 17β-OH group. In the $E_2$ docked to ERα model, H-bonds between $E_2$ and Glu[353], Arg[394], and His[524] side chains were observed (Fig. 5A). In the docked 17-hydroexemestane to ERα model (Fig. 5C), the same Arg[394] and His[524] interactions were maintained, except that there was a loss of the Glu[353] interaction. The R1881 docked to ERα model is shown in Supplementary Fig. S2A.[4]

Considering AR, the intermolecular interaction energy of 17-hydroexemestane was only 0.8 kJ/mol less favorable

than R1881, whereas exemestane was significantly less favorable than R1881 by 6.27 kJ/mol (Table 1). Docking of 17-hydroexemestane to AR, compared with the parent drug exemestane, indicated that 17-hydroexemestane exhibited improved lipophilic interactions by −2.11 kJ/mol, more favorable H-bonding interactions by −2.65 kJ/mol, and decreased steric clash by −1.08 kJ/mol. Hence, the 17β-OH group in 17-hydroexemestane compared with the 17-keto group in exemestane contributed −5.47 kJ/mol toward higher affinity for binding AR (Table 1). In the R1881 docked to AR model, H-bonds between R1881 and Asn[705], Gln[711] and Arg[752] were observed (Fig. 5B). The OH side chain of Thr[877] was in close proximity to both docked R1881 (Fig. 5B) and 17-hydroexemestane (Fig. 5D), but the angle was not favorable for H-bonding. Docking of 17-hydroexemestane to AR (Fig. 5D) indicated a short 2.78-Å H-bond between the 17β-OH group of the ligand and Asn[705], but not between the 3-keto group of the ligand and Gln[711] and Arg[752]. Hence, the short 2.78-Å H-bond observed in the 17-hydroexemestane docked to AR model was important in mediating high affinity binding. The exemestane docked to AR model is shown in Supplementary Fig. S2B.[4]

## Discussion

We observed that 17-hydroexemestane, the primary metabolite of exemestane, bound to ERα as a very weak ligand and acted through ER at high sub-micromolar and micromolar concentrations to stimulate growth, promote cell cycle progression, induce ERE-regulated reporter gene expression, and down-modulate ERα protein levels in breast cancer cells. However, we also observed that 17-hydroexemestane bound to AR as a strong ligand and found in T47D cells that 17-hydroexemestane stimulated growth, induced cell cycle progression, down-modulated AR mRNA expression, and stabilized AR protein levels, with all of these effects occurring at low nanomolar concentrations and blocked by bicalutamide. Moreover, computer docking indicated that the 17β-OH group of 17-hydroexemestane versus the 17-keto group of exemestane contributed significantly more toward increasing affinity to AR than to ERα. Molecular modeling also indicated that 17β-OH group of 17-hydroexemestane interacted with AR through an important H-bond of Asn[705], a conserved recognition motif employed by R1881. Therefore, we propose that the primary mechanism of action of exemestane *in vivo* is mediated by 17-hydroexemestane regulating AR activities.

The Food and Drug Administration label for exemestane (Aromasin; Pfizer) reports that in postmenopausal women with advanced breast cancer, the mean AUC (area under the curve) values of exemestane following repeated doses was 75.4 ng·h/mL (254 nmol·h/L), which was almost twice that in healthy postmenopausal women (41.4 ng·h/mL; 140 nmol·h/L; ref. 31). Because circulating levels of 17-hydroexemestane can reach about 1/10 the level of the parent compound (30), we hypothesize that circulating levels of 17-hydroexemestane are sufficient to bind AR and

regulate AR-dependent activities. Furthermore, a subpopulation of patients may exist who metabolize exemestane at higher rates, leading to correspondingly higher circulating 17-hydroexemestane levels. For instance, one of three patients administered 800 mg of exemestane, the highest dose evaluated, achieved 17-hydroexemestane plasma levels approximately one-half the level of the parent compound (30). Based on our results, we would predict that higher circulating levels of 17-hydroexemestane would associate with decreased rates of BMD loss and risk of bone fractures in postmenopausal women. We suggest that circulating levels of 17-hydroexemestane and exemestane should be determined in clinical trials and correlated to disease outcome and toxicity profiles such as BMD loss.

Although the clinical studies reported thus far were not designed to directly compare one AI versus another, comparisons in the rate of BMD loss from baseline to year 1, and from year 1 to 2 can be made. In the bone safety subprotocol of the IES (Intergroup Exemestane Study) trial,

the rate of BMD loss was greatest within 6 months of switching from tamoxifen to exemestane at −2.7% in the lumbar spine and −1.4% in the hip, but thereafter, BMD loss progressively slowed in months 6 to 12 and again in months 12 to 24 to only −1.0% and −0.8% in the lumbar spine and hip, respectively (10), which is in the same range as would be expected for postmenopausal women in general. However, in the bone safety substudy of the MA.17 trial, patients administered letrozole experienced a relatively constant rate of BMD loss for 2 years: at 12 months, the rate of BMD loss from baseline was −3.3% and −1.43% in lumbar spine and hip, respectively, and from year 1 to year 2, −2.05% and −2.17% in lumbar spine and hip, respectively (11). In the bone substudy of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial, the rate of BMD loss from baseline to year 1 was −2.2% in lumbar spine and −1.5% in hip and from year 1 to year 2, −1.8% in lumbar spine and −1.9% in hip (18). Collectively, these results suggest that after the initial



**Figure 5.** Intermolecular interactions of ligands complexed with ERα and AR by computer docking. **A,** superposition of E₂ from the X ray crystal structure (*gray*) and modeled E₂ (*yellow*) docked to ERα. **B,** superposition of R1881 from the crystal structure (*gray*) and modeled R1881 (*yellow*) docked to AR. **C,** modeled 17 hydroexemestane docked to ERα. **D,** modeled 17 hydroexemestane docked to AR. *Cyan, red,* and *blue,* hydrogen, oxygen, and nitrogen atoms, respectively. *Green,* carbon backbone of the protein. Hydrogens from the X ray crystal conformations of E₂ (**A**) and R1881 (**C**) were omitted. H bonds were shown to the modeled compound conformations only. *Dashed lines,* intermolecular H bonds up to 3.5 Å; their length in angstroms is indicated.

12 months of AI therapy, exemestane may be associated with slower rates of BMD loss compared with nonsteroidal AIs. Furthermore, although not directly comparable, the fracture rate per 1,000 woman-years in the ATAC trial was 22.6 for anastrozole and 15.6 for tamoxifen (1), whereas in the IES trial, the incidence rate per 1,000 woman-years for multiple fractures was 19.2 for exemestane and 15.1 for tamoxifen (10). These results show that although both anastrozole and exemestane were associated with higher fracture rates than tamoxifen, they also suggest that exemestane may be associated with a lower fracture rate than anastrozole. Clinical trials now under way to directly compare the different AIs will hopefully provide clear results.

Androgens regulate growth of normal and neoplastic mammary cells in a cell type-specific manner, either by inhibiting or stimulating growth (44). However, the mechanisms by which androgens via AR regulate breast cancer growth remain elusive. Female AR knock-out mice exhibit decreased ductal branching and terminal end buds in prepubertal animals and retarded lobuloalveolar development in adult animals (45). Likewise, targeted disruption of AR in MCF-7 cells also leads to severe inhibition of proliferation (45). Epidemiologic analyses indicate a positive correlation between androgen levels and the incidence of breast cancer; meta-analysis from nine prospective studies showed that a doubling in testosterone concentrations in postmenopausal women translated into an increased relative risk of 1.42 unadjusted and 1.32 adjusted for $E_2$ (46). AR status in breast cancer associates with both positive and negative indicators and clinical outcome. AR expression has been found in 84% (47) to 91% (48) of clinical breast cancers, and associated with ER status, but has also been found in 49% of ER-negative tumors (49). Patients with tumors that coexpress AR with ER and progesterone receptor have shown longer disease-free survival (DFS) than patients whose tumors were negative for all three receptors (48), but AR protein levels have also served as an independent predictor of axillary metastases in multivariate analysis (47) Furthermore, AR expression has correlated with decreased histopathologic grade, greater age, and postmenopausal status, but also lymph node positive status (50). In AR-positive/ER-negative tumors, AR expression again associated with positive and negative indicators/outcome such as increased age, postmenopausal status, and longer DFS but also tumor grade, tumor size, and HER-2/neu overexpression (49).

Patients who fail AI therapy, whether the AI was steroidal or nonsteroidal, likely harbor tumor cells that have been selected for growth in an estrogen-depleted environment and, hence, are not dependent on ER activity for survival. Not all androgens are metabolized by aromatase to estrogens; for instance, dihydrotestosterone cannot be converted to an estrogen by aromatase (44). Thus, a possible mechanism for failure of AI therapy in the clinic is androgen-stimulated breast cancer growth, a largely unrecognized alternative mechanism. We observed cellular proliferation of T47D cells in response to R1881 and 17-hydroexemestane, and these effects were blocked by bicalutamide. Therefore, T47D cells contain a functional AR signaling pathway that promoted growth in the absence of estrogen. Because functional AR signaling could be etiologically involved in a subpopulation of clinical breast cancers, those patients who have AR-positive tumors and achieve high circulating levels of 17-hydroexemestane, yet whose disease progresses while on exemestane therapy, may respond to AR-based therapy such as the antiandrogen bicalutamide.
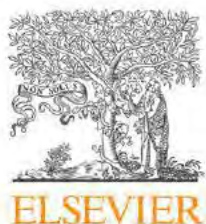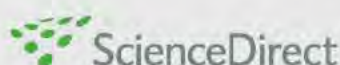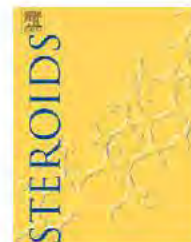
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Review

# New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer

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## ARTICLE INFO

## ABSTRACT

The metabolism of tamoxifen is being redefined in the light of several important pharmacological observations. Recent studies have identified 4-hydroxy N-desmethyltamoxifen (endoxifen) as an important metabolite of tamoxifen necessary for antitumor actions. The metabolite is formed through the enzymatic product of CYP2D6 which also interacts with specific selective serotonin reuptake inhibitors (SSRIs) used to prevent the hot flashes observed in up to 45% of patients taking tamoxifen. Additionally, the finding that enzyme variants of CYP2D6 do not promote the metabolism of tamoxifen to endoxifen means that significant numbers of women might not receive optimal benefit from tamoxifen treatment. Clearly these are particularly important issues not only for breast cancer treatment but also for selecting premenopausal women, at high risk for breast cancer, as candidates for chemoprevention using tamoxifen.

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## Contents

# 1.     Introduction

The aim of the body's biotransformation mechanisms is to prevent potentially toxic xenobiotic substances that include drugs, from damaging the body. That being the case, an orally active medicine must overcome numerous challenges to reach a target organ and produce the appropriate pharmacological effect at a receptor system. There is not one but several stages of biotransformation of a lipophilic drug such as tamoxifen that are designed to enhance the hydrophilic nature of the chemical so it can be rapidly eliminated. The stages of biotransformation are called phases I, II and III.

Phase I metabolism enhances the water solubility of a lipophilic chemical by hydroxylating an aromatic compound to become a phenol or hydrolyzing an esterified compound. These reactions are conducted by the family of cytochrome $P_{450}$ enzymes referred to as CYP's. Phase II metabolism further increases the water solubility of the Phase I product by attaching highly water soluble entities. In the case of selective estrogen receptor modulators (SERMs) sugars (glucuronic acid) and salts (sulfates) are the most important conjugation products. In contrast, the phase III system is efflux pump molecules (also known as *p*-glycoproteins and multi-drug resistance transports protein) that exclude unmetabolized drugs from the epithelial cells of the intestinal tract immediately upon absorption.

In general terms, the ingested SERM must survive "first pass" metabolism from the intestine to the liver to have any chance of reaching target organs around the body. The general principles are illustrated in Fig. 1 where the SERM is biotransformed by CYPs in the intestinal wall and Phase II metabolism occurs via intestinal bacteria. A fraction of the administered dose is then absorbed into the hepatic portal vein and further biotransformed by phase I CYPs and/or glucuronidated or sulfated in phase II metabolism in the liver. By way of example, only 2% of the administered raloxifene survives and is bioavailable for systemic distribution [1].

# 2.     Tamoxifen, the first SERM

The nonsteroidal antiestrogen tamoxifen (ICI 46,474 Nolvadex®) is a pioneering medicine [2] used to treat all stages of breast cancer in more than 120 countries throughout the world. The compound ICI 46,474 was discovered in the Fertility Control Program at Imperial Chemical Industries (ICI Pharmaceuticals Division, now AstraZeneca) in Alderley Park, Cheshire, England in the early 1960s [3–5]. The drug was found to be an extremely potent postcoital contraceptive in the rat [4,5]. Unfortunately, ICI 46,474 did not exhibit antifertility properties in women, in fact, quite the opposite, it induced ovulation [6,7]. As a result, the medicine was, at one time, marketed in the United Kingdom for the induction of ovulation in subfertile women with a functional hypothalamo-pituitary-ovarian axis.

There is a known link between estrogen and the initiation and growth of some breast cancers [8] so the nonsteroidal antiestrogen ICI 46,474 was tested as a potential treatment for advanced breast cancer in postmenopausal women. The antiestrogen produced response rates of 25–35% in unselected patients comparable to diethylstilbestrol and high dose androgen therapy, the standard endocrine therapies at the time [9,10]. However, fewer side effects were noted with tamoxifen [9,10]. As a result, the drug was approved as a palliative option for the hormonal treatment of breast cancer in the UK in 1973. There the story may have ended had not tamoxifen been reinvented as the first targeted therapy for breast cancer [2].

The seminal observations by Elwood Jensen that estrogen action is mediated by the estrogen receptor (ER) [11,12] in its target tissues (uterus, vagina, pituitary and breast tumors) opened the door to targeting tamoxifen to select patients with the ER in their metastatic tumor [13,14]. However, a strategic plan was developing to use tamoxifen in a broader range of patient populations. Laboratory studies conducted in the 1970s showed that tamoxifen blocked estrogen binding to the ER [15–17], should be used as a long-term adjuvant therapy to suppress tumor recurrence [18–20] and the drug also had potential as a chemopreventive agent [21,22].

Clinical studies subsequently confirmed that long-term adjuvant tamoxifen therapy, targeted to the patients with ER positive breast cancers, significantly decreased the death rate from the disease [23] and contributes to the current decline in death from breast cancer nationally [24]. Overall, the strategy of targeted long-term "antiestrogenic" [25] treatment for breast cancer has presaged the current fashion of targeting anticancer agents to other organ sites in the body.

Despite the fact that aromatase inhibitors show superiority over tamoxifen as adjuvant therapy in postmenopausal women [26–29], several issues have surfaced that have retained tamoxifen as a useful therapeutic agent worldwide. The medicine is extremely cheap compared to aromatase inhibitors so tamoxifen remains an essential anticancer agent in undeveloped countries or in countries with under-funded managed healthcare systems. Furthermore, tamoxifen is the only appropriate antiestrogenic therapy for premenopausal women whether they are being treated for breast cancer or whether chemoprevention is being considered [30]. For these reasons, new knowledge that can enhance the appropriate use of an established drug is of value to improve healthcare.

There are current initiatives to translate emerging knowledge on genetic variations in drug metabolism to target patient populations [31]. It is reasoned that by applying pharmacogenomic tests to specific patient populations, there will be fewer surprises with side effects, drug interactions, and a higher probability of increasing therapeutic effectiveness in the treatment or prevention of disease. The promise of practical progress is exemplified in this article using tamoxifen as the model drug.

Tamoxifen is a prodrug and can be metabolically activated to 4-hydroxytamoxifen [32–34] or alternatively can be metabolically routed via N-desmethyltamoxifen to 4-hydroxy-N-desmethyltamoxifen [35,36] (Fig. 2). The hydroxy metabolites of tamoxifen have a high binding affinity for the ER [32,37]. The finding that the enzyme produced by CYP2D6 activates tamoxifen to hydroxylated metabolites 4-hydroxytamoxifen and endoxifen [38] has implications for cancer therapeutics. Women with enzyme variants that cannot make endoxifen may not have as successful an outcome
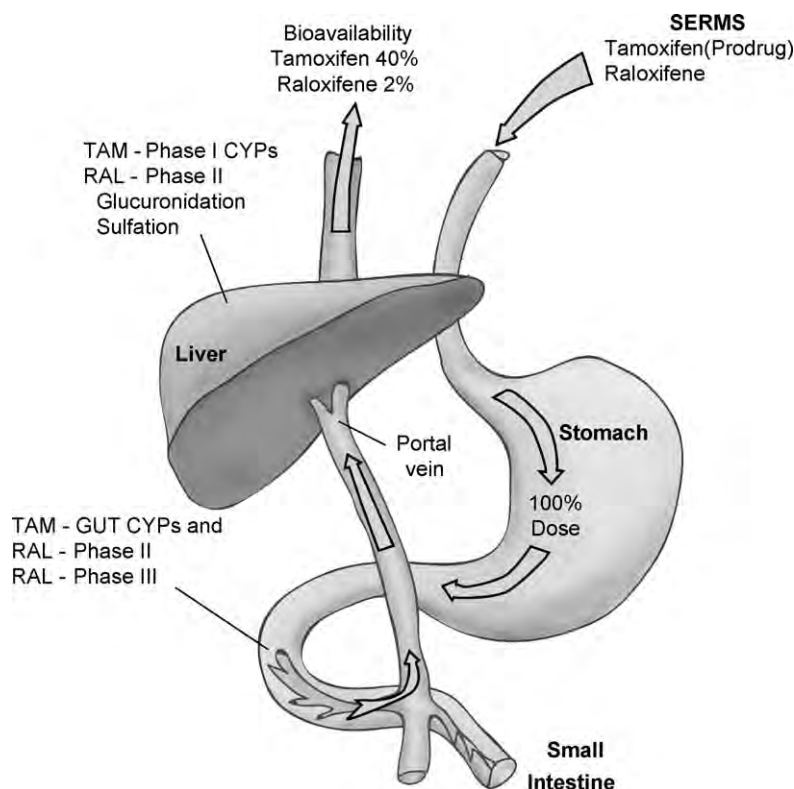
**Fig. 1 – The stylized representation of the absorption of two selective estrogen receptor modulators (SERMS) tamoxifen (TAM) or raloxifene (RAL) into the circulation as bioactive molecules. The polyphenolic SERM raloxifene must transverse phase II and phase III obstacles in the gut and the liver to get into the general circulation. This results in very little of the ingested drug being bioavailable at target sites. In contrast, tamoxifen is extremely lipophilic and 98% protein bound to serum albumin. This extends the duration of action of tamoxifen because phase II metabolism to phenolic compounds is retarded.**

with tamoxifen therapy. Alternatively, women who have a normal enzyme may make high levels of the potent antiestrogen endoxifen and experience hot flashes. As a result, these women may take selective serotonin reuptake inhibitors (SSRIs) to ameliorate hot flashes but there are potential pharmacological consequences to this strategy. Some of the SSRIs are metabolitically altered by the *CYP2D6* enzyme product [39]. It is therefore possible to envision a drug interaction whereby SSRIs block the metabolic activation of tamoxifen.

This article will describe the scientific twists and turns that tamoxifen and its metabolites have taken over the past 30 years. The story is naturally dependent on the fashions in therapeutic research at the time. What seems obvious to us as a successful research strategy today, with millions of women taking tamoxifen, was not so 30 years ago at the beginning when the clinical community and pharmaceutical industry did not see "antihormones" as a priority at all for drug development [25]. In 1972, tamoxifen was declared an orphan drug with no prospects [2].

## 3. Basic mechanisms of tamoxifen metabolism

The original survey of the putative metabolites of tamoxifen was conducted in the laboratories of ICI Pharmaceuticals Divi-

sion and published in 1973 [40]. A number of hydroxylated metabolites were noted (Fig. 3) following the administration of $^{14}C$ labeled tamoxifen to various species (rat, mouse, monkey, and dog). The major route of excretion of radioactivity was in the feces. The rat and dog were used to show that up to 53% of the radioactivity derived from tamoxifen was excreted via the bile and up to 69% of this was reabsorbed via a enterohepatic recirculation until eventual elimination occurs [40]. The hydroxylated metabolites are excreted as glucuronides. However, no information about their biological activity was available until the finding that 4-hydroxytamoxifen had a binding affinity for the ER equivalent to 17β estradiol [32]. Similarly, 3,4-dihydroxytamoxifen (Fig. 3) bound to the human ER but interestingly enough, 3,4-dihydroxytamoxifen was not significantly estrogen-like in the rodent uterus despite being antiestrogenic [32].

Additional studies on the metabolism of tamoxifen in four women [41] identified 4-hydroxytamoxifen as the primary metabolite using a thin layer chromatographic technique to identify $^{14}C$ labeled metabolites. This assumption, coupled with the potent antiestrogenic actions of 4-hydroxytamoxifen [32] and the conclusion that it was an advantage, but not a requirement for tamoxifen to be metabolically activated [33,42] seemed to confirm the idea that 4-hydroxytamoxifen was the active metabolite that bound in rat estrogen target tissues to block estrogen action [34]. However, the origi-
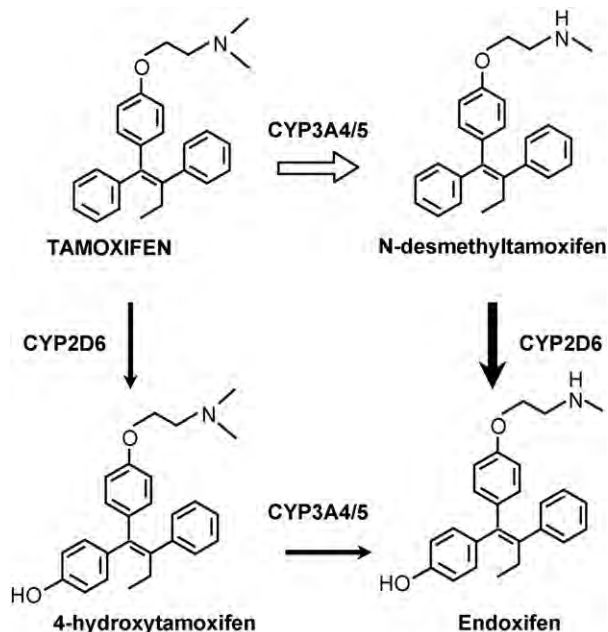
**Fig. 2 – The metabolic activation of tamoxifen to phenolic metabolites that have a high binding activity for the human estrogen receptor. Both 4-hydroxytamoxifen and endoxifen are potent antiestrogens *in vitro*.**

nal analytical methods used to identify 4-hydroxytamoxifen as the major metabolite in humans were flawed [43] and subsequent studies identified N-desmethyltamoxifen (Fig. 4) as the major metabolite circulating in human serum [44]. The metabolite was found to be further demethylated to N-desdimethyltamoxifen (metabolite Z) [45] and then deaminated to metabolite Y, a glycol derivative of tamoxifen [46,47].

The metabolites (Fig. 4) that are not hydroxylated at the 4 position of tamoxifen (equivalent to the three phenolic hydroxyl of estradiol) are all weak antiestrogens that would each contribute to the overall antitumor actions of tamoxifen at the ER based on their relative binding affinities for the ER and their actual concentrations locally.

At the end of the 1980s the identification of another metabolite tamoxifen 4-hydroxy N-desmethyltamoxifen in animals [48] and man [35,36] was anticipated but viewed as obvious and uninteresting. The one exception that was of interest was metabolite E (Fig. 3) identified in the dog [40]. This phenolic metabolite without the dimethylaminoethyl side chain is a full estrogen [47,49]. The dimethylaminoethoxy side chain of tamoxifen is necessary for antiestrogenic action [49].

It is not a simple task to study the actions of metabolites *in vivo*. Problems of pharmacokinetics, absorption and subsequent metabolism all conspire to confuse the interpretation of data. Studies *in vitro* using cell systems of estrogen target tissues were defined and refined in the early 1980s to create an understanding of the actual structure–function relationships of tamoxifen metabolites. Systems were developed to study the regulation of the prolactin gene in primary cultures of immature rat pituitary gland cells [42,50] or cell replication in ER positive breast cancer cells [51–54]. Overall, these models were used to describe the importance of a phenolic hydroxyl to tether a triphenylethylenes appropriately in the ligand-binding domain of the ER and to establish the appropriate positioning of an "antiestrogenic" side chain in the "antiestrogen region" of the ER [50] to modulate gene activation and growth [42,50,55–58]. These structure–function studies, that created hypothetical models of the ligand-ER/complex, were rapidly advanced with the first reports of the X-ray crystallography of the estrogen, 4-hydroxytamoxifen [59] or raloxifene ER [60] complexes. The ligand–receptor protein interaction
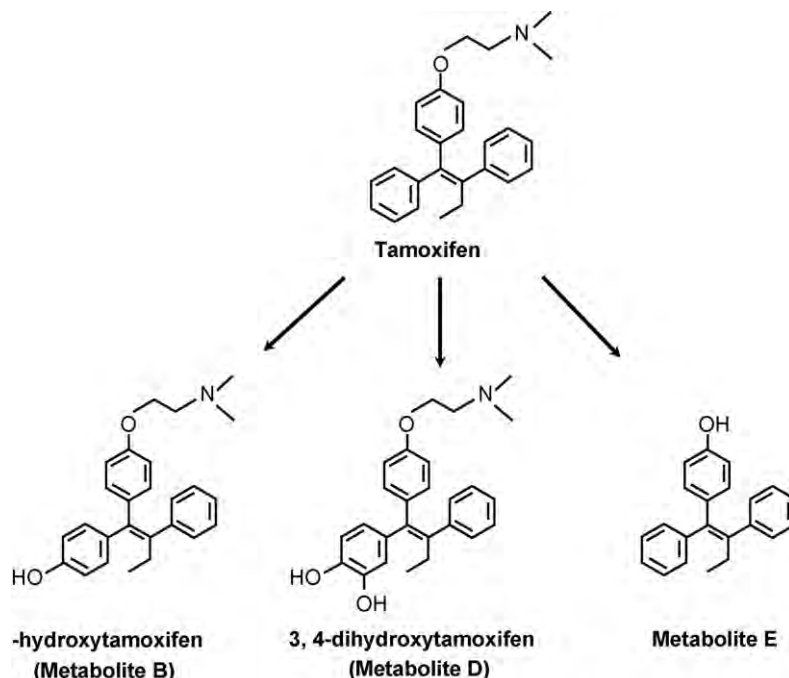


**Fig. 3 – The original hydroxylated metabolites of tamoxifen noted in animals by Fromson et al. [40].**
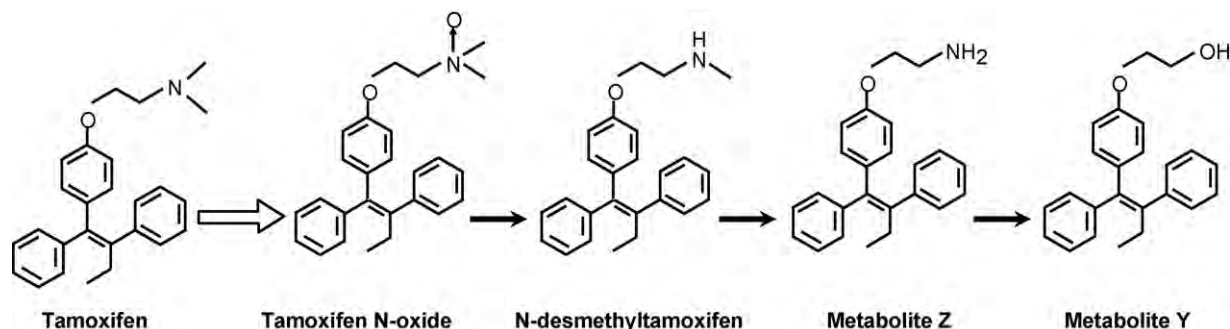
**Fig. 4** – The serial metabolic demethylation and deamination of the antiestrogenic side chain of tamoxifen. Each of the metabolites is a weak antiestrogen with poor binding affinity for the estrogen receptor.

was subsequently interrogated by examining the interaction of the specific amino acid, asp 351 with the antiestrogenic side chain of the ligand [61]. A mutation was found as the dominant ER species in a tamoxifen-stimulated breast tumor grown in athymic mice [61,62]. The structure–function relationships studies, that modulated estrogen action at a transforming growth factor alpha gene target, demonstrated that the ligand shape would ultimately program the shape of the ER complex in a target tissue [30,63–65]. This concept is at the heart of metabolite pharmacology and is required to switch on and switch off target sites around the body. The other piece of the mechanism of SERMs puzzle that was eventually solved was the need for another player to partner with the ER complex. Coactivators [66] can enhance the estrogen-like effects of compounds at a target site [67]. However, in the early 1990s, the molecular and clinical use of this knowledge with the development and application of SERMs was in the future [68].

The urgent focus of translational research in the early 1990s was to discover why tamoxifen was a complete carcinogen in rat liver [69,70] and to determine whether there was a link between metabolism and the development of endometrial cancer noted in very small but significant numbers of postmenopausal women taking adjuvant tamoxifen [71,72].

All interest in the metabolism of tamoxifen focused on the production of DNA adducts [73] that were responsible for rat liver carcinogenesis and, at the time, believed to be poten-

tially responsible for carcinogenesis in humans [74]. Although many candidates were described [75–78], the metabolite found to be responsible for the initiation of rat liver carcinogenesis is α-hydroxytamoxifen [79–83] (Fig. 5). α-Hydroxytamoxifen has been resolved into R-(+) and S-(−) enantiomers. Metabolism by rat liver microsomes gave equal amounts of the two forms, but in hepatocytes the R form gave 8× the level of DNA adducts as the S form. As both had the same chemical reactivity towards DNA, Osborne et al. [84] suggested that the R form was a better sulfotransferase substrate. This enzyme is believed to catalyze DNA adduct formation. Subsequently, Osborne et al. [85] conducted studies with alpha-hydroxy-N-desmethyltamoxifen; the R-(+) gave 10× the level of adducts in rat hepatocytes as the S-(−).

There were reasonable concerns that the hepatocarcinogenicity of tamoxifen in rats would eventually translate to humans but fortunately this is now known to be untrue [86]. The demonstration of carcinogenesis in the rat liver appears to be related to poor DNA repair mechanisms in the inbred strains of rats. In contrast, it appears that the absence of liver carcinogenesis in women exposed to tamoxifen [87] is believed to result from the sophisticated mechanisms of DNA repair inherent in humans cells.

It is clear from this background about the early development of tamoxifen and the fact that tamoxifen was considered to be such a safe drug in comparison to other cytotoxic agents
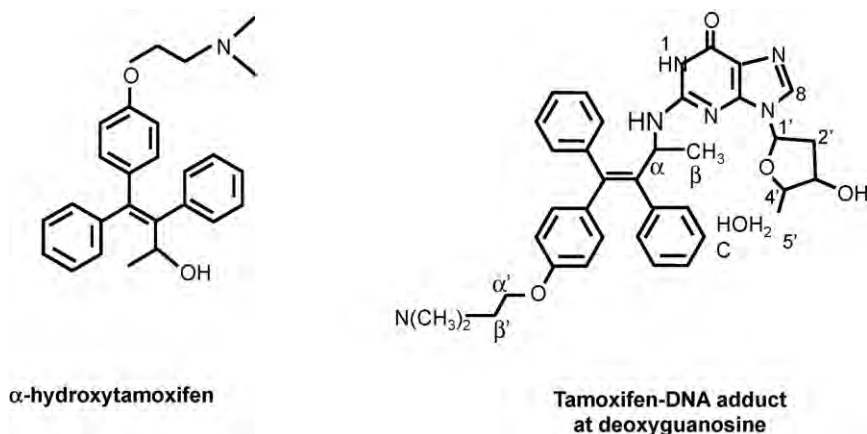


**Fig. 5** – The putative metabolite of tamoxifen, α-hydroxytamoxifen that produces DNA adducts through covalent binding to deoxyguanosine.
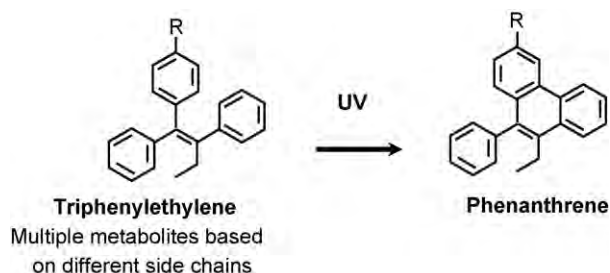
**Fig. 6 – The UV activation of a triphenylethylenes to a florescent phenanthrene. This basic reaction is exploited in the detection of serum tamoxifen levels.**

used in therapy during the 1970s and 1980s, that there was little enthusiasm for in-depth studies of tamoxifen metabolism. However, this perspective was to change in the 1990s with the widespread use of tamoxifen as the gold standard for the treatment and prevention of breast cancer. Questions needed to be addressed: (1) what happens to tamoxifen in patients? and (2) can improvements be made to the molecule?.

## 4.    Clinical pharmacology

A number of analytical techniques are available to evaluate blood levels of tamoxifen and its metabolites once the drug is absorbed. The early method of thin layer chromatography, and the current method of high performance liquid chromatography (HPLC) both depend on the conversion of the triphenylethylenes to fluorescent phenanthrenes for their detection (Fig. 6). The original description of the reaction [88] was successfully adapted [89] to identify tamoxifen, N-desmethyltamoxifen and 4-hydroxytamoxifen in plasma samples.

Subsequent improvements were made [90] but the method significantly underestimated phenolic metabolites (4-hydroxytamoxifen) and had no internal standardization. In contrast, a method of post-column fluorescence activation [91] or preliminary purification from interfering substance using a Sep-Pack C18 cartridge (Waters Association, Milford, MA) [92] with internal standardization considerably improved accuracy. The detection of tamoxifen metabolites in serum was further improved by Lien et al. [93] and recently by Lee et al. [94] who adapted the methods [95,96] developed to perform "on line" extraction and post-column cyclization. Using this methodology the limits of detection for 4-hydroxy tamoxifen and endoxifen are 0.5 and 0.25 ng/ml, respectively [97]. Since there was such initial controversy about the identification of metabolites in patient serum, it is perhaps important to describe the validation of 4-hydroxy-desmethyltamoxifen as a metabolite of tamoxifen in patients. Tamoxifen metabolites were investigated in a 57-year-old female patient receiving tamoxifen treatment [35]. Two major chromatographic peaks were identified in bile following treatment with β-glucuronidase. On major peak co-eluted with 4-hydroxytamoxifen but the second peak was proven to be 4-hydroxy-N-desmethyltamoxifen using (a) co-elution with an authentic standard on reversed-phase chromatography

and formation of fluorescent derivative by cyclization; (b) the detection of a molecular ion (M+l)+ of 374 m/2 as determined by liquid chromatography–mass spectrometry; and (c) a fragmatogram identical to that of the authentic standard, obtained by mass spectrometry. Subsequent refinement of the technology improved detection for identification of 4-hydroxy-N-desmethyltamoxifen in human serum, tissues [36] and rat tissues [93].

Studies confirm that tamoxifen is 98% bound to serum albumin which ultimately creates a long biological half-life (plasma half-life 7 days) [93]. A single oral dose of 10 mg tamoxifen (half the daily dose) produces peak serum levels of 20–30 ng of tamoxifen/ml within 3–6 h but it must be stressed that patient variation is very large [98]. Nevertheless, continuous therapy with either 10 mg bid [98] or 20 mg bid [99] produces steady state levels within 4 weeks. Blood levels of tamoxifen can average around 150 ng/ml for 10 mg tamoxifen bid and 300 ng/ml for 20 mg tamoxifen bid. A strategy of using loading doses [98,100] to elevate blood levels rapidly has not produced any therapeutic benefit.

Overall, the results from the metabolic studies with tamoxifen during the 1970s and 1980s did not help clinicians to use tamoxifen more effectively. The structures of metabolites were in fact used as leads to create new molecules for clinical development.

## 5.    Metabolic mimicry

The demonstration [32] that the class of compounds referred to as nonsteroidal antiestrogens were metabolically activated to compounds with high binding affinity for the ER created additional opportunities for the medicinal chemists within the pharmaceutical industry to develop new agents. This was particularly true once the nonsteroidal antiestrogens were recognized to be SERMs [101–103] and had applications not only for the treatment and prevention of breast cancer but also as potential agents to treat osteoporosis and coronary heart disease [104,105]. The reader is referred to other recent review articles to obtain further details of new medicines under investigation [104,105] but some current examples are worthy of note and will be mentioned briefly. Compounds of interest that have their structural origins as metabolites from nonsteroidal antiestrogens are summarized in Fig. 7. Raloxifene is an agent that originally was destined to be a drug to treat breast cancer but it failed in that application [106]. It appears that the pharmacokinetics and bioavailability of raloxifene are a challenge. Only about 2% of administered raloxifene is bioavailable [1] but despite this, the drug is known to have a long biological half-life of 27 h. The reason for this disparity is that raloxifene is a polyphenolic drug that can be glucuronidated and sulfated by bacteria in the gut so the drug cannot be absorbed [107,108]. This phase II metabolism in turn controls enterohepatic recirculation and ultimately impairs the drug from reaching and interacting with receptors in the target. This concern has been addressed with the development of the long-acting raloxifene derivative arzoxifene that is known to be superior to raloxifene as a chemopreventive in rat mammary carcinogenesis [109]. One of the phenolic groups (Fig. 7) is methylated to provide protection from phase II metabolism.
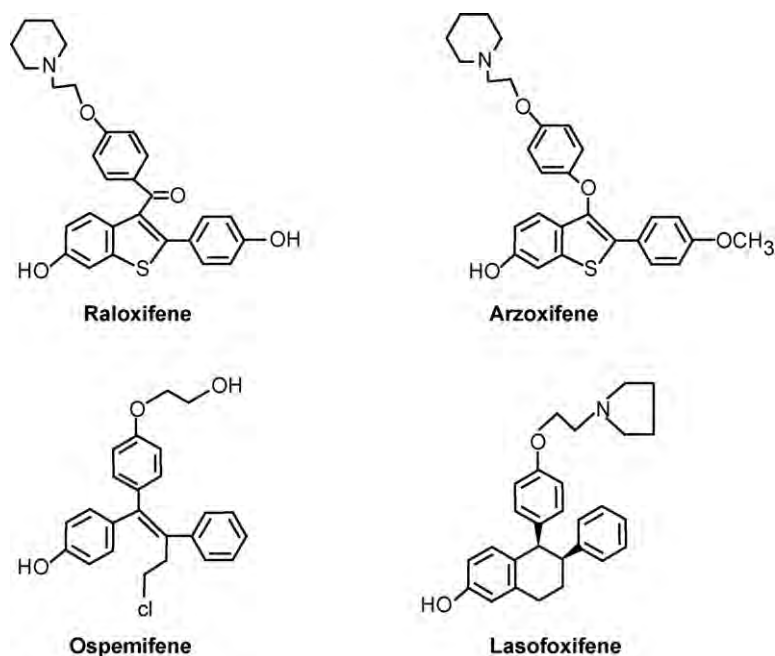
**Fig. 7 – The formulae of SERMs that have been developed based on the knowledge of the metabolic activation of tamoxifen (and nafoxidine, see text) as well as the metabolism of the antiestrogen side chain of tamoxifen to a glycol.**

Nevertheless, arzoxifene has not performed well as a treatment for breast cancer [110,111]; higher doses are less effective than lower doses. These data imply that effective absorption is impaired by phase III metabolism. That being said, the results of trials evaluating the effects of arzoxifene as a drug to treat osteoporosis, using lower doses, are eagerly awaited. Perhaps arzoxifene will be a better breast cancer preventive than a treatment.

Unfortunately, the bioavailability of phenolic drugs is also dependent on phase II metabolism to inactive conjugates in the target tissue. 4-Hydroxytamoxifen [32] is only sulfated by three of seven sulfotransferase isoforms whereas raloxifene is sulfated by all seven [112]. Maybe local phase II metabolism plays a role in neutralizing the antiestrogen action of raloxifene in the breast. Falany et al. [112] further report that SULT1E1, that sulfates raloxifene in the endometrium, is only expressed in the secretory phase. In contrast, 4-hydroxytamoxifen is sulfated at all stages of the uterine cycle.

Lasofoxifene is a diaryltetrahydronaphthalene derivative referred to as CP336156 [113] that has been reported to have high binding affinity for ER and have potent activity in preserving bone density in the rat [114,115]. The structure of CP336156 is reminiscent of the putative antiestrogenic metabolite of nafoxidine [116] that failed to become a breast cancer drug because of unacceptable side effects [117]. There are two disasterometiric salts of the chemical shown in Fig. 7. CP336156 is the *l* enantiomer that has 20 times the binding affinity for the ER as the *d* enantiomer. Studies demonstrate that the *l* enantiomer had twice the bioavailability of the *d* enantiomer. The authors [113] ascribed the difference to enantioselective glucuronidation of the *d* isomer. An evaluation of CP336156 in the prevention and treatment of rat mammary tumors induced by N-nitroso-N-methylurea shows activity similar to that of tamoxifen [118].

Ospemifene or deaminohydroxytoremifene is related to metabolite Y formed by the deamination of tamoxifen [47]. Metabolite Y has a very low binding affinity for the ER [47,119] and has weak antiestrogenic properties compared with tamoxifen. Ospermifene is a known metabolite of toremifene (4 chlorotoremifene) but unlike tamoxifen, there is little carcinogenic potential in animals [120]. It is possible that the large chlorine atom on the 4 position of toremifene and ospermifene reduces α hydroxylation to the ultimate carcinogen related to α hydroxy tamoxifen (Fig. 6). Deaminohydroxytoremifene has very weak estrogenic and antiestrogenic properties *in vivo* [121] but demonstrates SERM activity in bone and lowers cholesterol. The compound is proposed to be used as a preventative for osteoporosis. Preliminary clinical data in healthy men and postmenopausal women demonstrate pharmacokinetics suitable for daily dosing between 25 and 200 mg [122]. Interestingly enough, unlike raloxifene, ospermifene has a strong estrogen-like action in the vagina but neither ospermifene nor raloxifene affect endometrial histology [123,124]. Overall, the goal of developing a bone specific agent is reasonable, but the key to commercial success will be the prospective demonstration of the prevention of breast and endometrial cancer as beneficial side effects. This remains a possibility based on prevention studies completed in the laboratory [125,126].

## 6. Tamoxifen metabolism today

A comprehensive evaluation of the sequential biotransformation of tamoxifen has been completed by Desta et al. [38]. They used human liver microsomes and experiments with specifically expressed human cytochrome P450's to identify the prominent enzymes involved in phase I metabolism. Their

results are summarized in Fig. 2 with the relevant CYP genes indicated for the metabolic transformations. The authors make a strong case that N-desmethyltamoxifen, the principal metabolite of tamoxifen that accumulates in the body, is converted to endoxifen by the enzymatic product of CYP2D6. The CYP2D6 product is also important to produce the potent primary metabolite 4-hydroxytamoxifen but the metabolite can also be formed by the enzymatic products: CYP2B6, CYP2C9, CY2C19 and CYP3A4.

The CYP2D6 phenotype is defined as the metabolic ratio (MR) by dividing the concentration of an unchanged probe drug, known to be metabolized by the CYP2D6 gene product, by the concentration of the relevant metabolite at a specific time. These measurements have resulted in the division of the CYP2D6 phenotype in four metabolic classes: poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM) and ultrarapid metabolizes (UM). Over 80 different single nucleotide polymorphisms have been identified but there are inconsistencies in the precise definitions of the ascribing a genotype to a phenotype [127,128]. Bradford [128] and Raimundo et al. [129] have described the frequency of common alleles for CYP2D6. Pertinent to the current discussion of tamoxifen metabolism, the CYP2D6*4 allele [130] is estimated to have a frequency of 12–23% in Caucasians, 1.2–7% in black Africans and 0–2.8% in Asians [127,128]. A lower estimate of (<10%) of the PM phenotype is presented by Bernard et al. [131].

The molecular pharmacology of endoxifen has recently been reported [37,132,133]. Endoxifen and 4-hydroxytamoxifen were equally potent at inhibiting estrogen-stimulated growth of ER positive breast cancer cells MCF-7, T47D and BT474. Both metabolites are significantly superior in vitro to tamoxifen the parent drug. Additionally, the estrogen-responsive genes pS$_2$ and progesterone receptor were both blocked to an equivalent degree by endoxifen and 4-hydroxytamoxifen [132,133]. Lim et al. [133] have extended the comparison of endoxifen and 4-hydroxytamoxifen in MCF-7 cells by comparing and contrasting global gene regulation using the Affymetrix U133A Gene Chip Array. There were 4062 total genes that were either up or down regulated by estradiol whereas, in the presence of estradiol, 4-hydroxytamoxifen or endoxifen affected 2444 and 2390 genes, respectively. Overall, the authors [133] demonstrated good correlation between RTPCR and select genes from the microarray and concluded that the global effects of endoxifen and 4-hydroxytamoxifen were similar.

Stearns et al. [97] and Jin et al. [134] have confirmed and significantly extended Lien's original identification of endoxifen and observation [35,36] that there are usually higher circulating levels of endoxifen than 4-hydroxytamoxifen in patients receiving adjuvant tamoxifen therapy. However, Flockhart's group [97] have advanced the pharmacogenomics and drug interactins surrounding tamoxifen therapy that should be a consideration in the antihormonal treatment of breast cancer.

The ubiquitous use of tamoxifen for the treatment of node negative women [135] during the 1990s, the use of tamoxifen plus radiotherapy following lumpectomy for the treatment of ductal carcinoma in situ (DCIS) [136] as well as the option to use tamoxifen for chemoprevention in high risk pre- and postmenopausal women [137] enhanced awareness of the menopausal side effects experienced by women when taking tamoxifen. Up to 45% of women with hot flashes grade them as severe [137] therefore there have been efforts to improve quality of life. Treatments with the SSRIs are popular [97,138,139] (Fig. 8). The SSRIs are twice as effective as the "placebo" effect at reducing menopausal symptoms in randomized clinical trials [138–140], so there is naturally an increased usage of SSRIs with long-term tamoxifen treatment to maintain compliance. Unfortunately, the metabolism of tamoxifen to hydroxylated metabolites [141–143] and the metabolism of SSRIs [39,144–147] both occur via the CYP2D6 gene product. Indeed Stearns et al. [97] showed that the SSRI inhibitor paroxetine reduced the levels of endoxifen during adjuvant tamoxifen
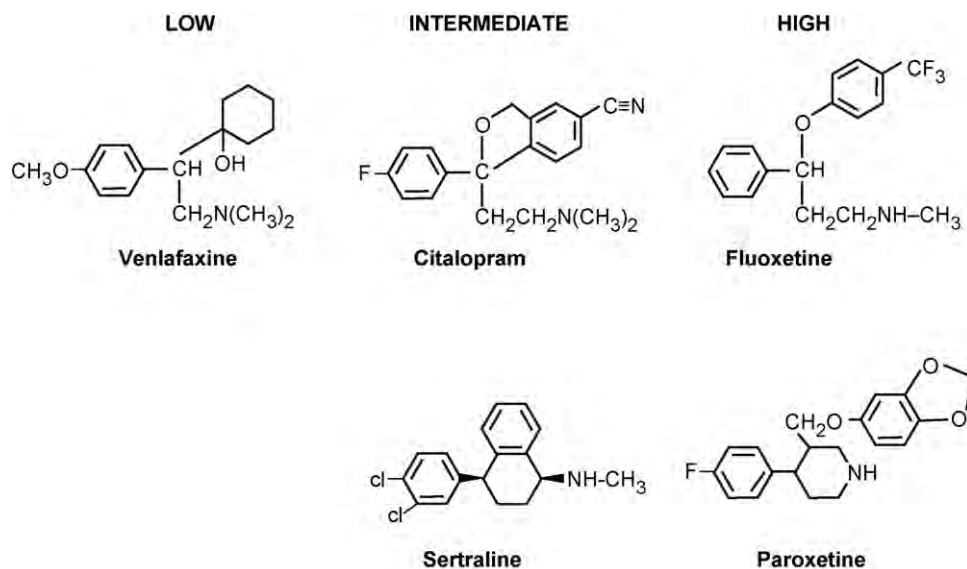


Fig. 8 – The structures of selective serotonin reuptake inhibitors (SSRIs) that have low intermediate or high affinity for the CYP2D6 enzyme system. High affinity binders for CYP2D6 block the metabolic activation of tamoxifen to endoxifen (Fig. 2).

therapy and endoxifen levels decrease by 64% in women with wild type CYP2D6 enzyme. Patients were examined who were taking venlafaxine, sertraline, and paroxetine and compared with those women who were homozygotes for the CYP2D6*4/*4 inactive genotype. Patients with the wild type gene who took the most potent inhibitor paroxetine had serum levels of endoxifen equivalent to the patients with the aberrant CYP2D6 gene. In fact, the clinical data were consistent with the inhibition constants for the inhibition of CYP2D6 by paroxetine (potent), fluoxetine, sertraline, citalopram (intermediate) and venlafaxine (weak) which are 0.05, 0.17, 1.5, 7 and 33 µmol/l, respectively.

The CYP2D6 gene product that is fully functional (wild type) is classified as the CYP2D6*1. A large number of alleles are associated with no enzyme activity or reduced activity. Conversely, high metabolizers can have multiple copies of the CYP2D6 allele [31]. A recent study by Borges et al. [148] continues to expand our understanding of the detrimental effect of CYP2D6 variants plus concomitant administration of SSRIs on endoxifen levels. But, it is the clinical correlations with tumor responses and side effects that are starting to provide clues about the importance of pharmacogenomics for tamoxifen to be optimally effective as a breast cancer drug.

## 7.　Clinical correlations

The significance of genotyping on clinical outcomes of a tamoxifen trial have been addressed using paraffin-embedded tumor blocks from a North Central Center Treatment Group (NCCTG) trial NCCTG 89-30-52 [149]. The postmenopausal women with ER positive tumors received 5 years of adjuvant tamoxifen therapy. The tumor blocks were used to determine CY2D6 (*4 and *6) and CYP3A5 (*3) and 17 buccal swabs were used to test the veracity of the tumor genotyping. The concordance rate for the buccal swabs was 100%. Overall, the CYP3A5*3 variant was not associated with any adverse clinical outcomes but the women with the CYP2D6*4/*4 genotype had a higher risk of disease relapse but a lower incidence of side effects such as hot flashes [149]. The implication is that tamoxifen must be converted to endoxifen, a more potent antiestrogen.

In a follow up study [150] using the same database established for trial NCCTG 89-30-52, patient records were screened to determine the extent of SSRI prescribing. The goal was to establish the combined effect of genotyping and SSRI inhibition of the CYP2D6 enzyme. Overall, the authors [150] concluded that a mutated CYP2D6 gene or the inadvertent use of SSRIs that inhibit the CYP2D6 enzyme product are independent predictors of breast cancer outcomes for postmenopausal women with breast cancer taking tamoxifen. In a recent complimentary study, Mortimer et al. [151] demonstrated that hot flashes were a strong predictor of positive outcomes for adjuvant tamoxifen treatment.

Although all of the current emphasis has been on the biological effects of tamoxifen in patients with the CYPD6*4 variant, studies of CYP3A5* 1 and *3 1A1 *1 and 2 and UGT2B15 * and *2 have been undertaken and compared with car-

riers of CYP2D6*4. In contrast to the studies of Goetz et al. [149], patients who carry the SULT1A1*1, CYP2D7*4 and CYP3A5*3 alleles, and would be predicted to give rise to lower concentrations of metabolites with high affinity for the ER, might actually benefit from tamoxifen [152–155]. No differences were noted between genotypes CYP2D6, SULT1A1 or UGT 2B15 and tamoxifen treatment but Wegman et al. [155] claim that genetic variants of CYP3A5 may predict response to tamoxifen. Clearly, reasons for the different conclusions need to be advanced. The hypothesis that variants of metabolizing enzymes can affect patient outcomes for the treatment of breast cancer must now be addressed in large populations and with prospective studies.

## 8.　Conclusions

Overall, the study of tamoxifen metabolism has provided important clues which guided medicinal chemists to synthesize and develop new medicines. The study of metabolites has also provided valuable insight into the mechanism of action of SERMs at their target the ER. However, it is the recent research on the value of genotyping CYPs in breast cancer patients to improve response rates to tamoxifen therapy that is showing important promise. Genotyping patients for CYP2D6 appears to be valuable to exclude the suboptimal use of tamoxifen in select individuals. Additionally, and perhaps more importantly, an effect of SSRIs on the blood levels of endoxifen has raised the possibility that the cheap and effective veteran tamoxifen could be targeted further to select populations of women to improve response rates. Avoiding SSRIs with a high affinity for CYP2D6 gene product could improve tamoxifen's efficacy. Since tamoxifen is still the antihormonal treatment of choice for premenopausal patients and the only choice for breast cancer risk reduction in premenopausal women, then genotyping from buccal swabs appears to be a cheap and effective way of ensuring that tamoxifen is used to treat the appropriate woman.

It is necessary, however, to close on a note of caution. Very few patients have been studied to create definitive guidelines. That being said, the task of proving the value of these tantalizing clues and hypotheses is the responsibility of clinicians to organize prospective clinical trials or at least there must be investment in the further analysis of archival material from randomized trials. The value of committing resources to establish hypothesis as fact is clear. An important cheap medicine should potentially be given only to women who will benefit from it. Indeed, it may be the role of CYP2D6 in tamoxifen metabolism that is creating the small but significant advantage of aromatase inhibitors versus tamoxifen in postmenopausal women [26,27]. Again, this can be tested as the tumor blocks and patient records could be reviewed to determine genotyping and whether SSRIs were used. It would be remarkable to discover that the pharmacology of tamoxifen is undermining activity rather than the current view that aromatase inhibitors were better medicines because they have, unlike the SERMs, no estrogen-like actions at the level of the tumor.

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*Point/Counterpoint*

# Tamoxifen or Raloxifene for Breast Cancer Chemoprevention: A Tale of Two Choices—Point

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The stated goal for an investment in cancer research is the eradication of cancer. But this is just talk. A world with no cancer is a noble goal but the problem becomes where to start. In other words, how to put ideas into action and move forward from rhetoric. The task is enormous, but one solution where there has been much talk is cancer prevention. In the case of lung cancer, the solution is simple stop smoking. But the social engineering that is required to prevent one sector of society from creating a massive health care crisis in another seems to be insoluble. It is now clear that women have been the victims here through a callous campaign to recruit smokers. Lung cancer is the disease that kills more women with cancer than any other. Based on this inconvenient truth of modern society, is there any reason to believe that the cancer research community has made any progress with practical help for people? In contrast to lung cancer, progress is quantifiable in another major killer of women breast cancer.

In 1971, President Nixon signed the National Cancer Act and declared war on cancer, but there were no serious plans to prevent breast cancer. Nevertheless, the first experiments were being conducted to prevent breast cancer with antihormones but, regrettably, at that time no one cared (1). All efforts were focused on the application of combinations of cytotoxic chemotherapy to treat and cure cancer by killing the last cancer cell. Despite heroic attempts to kill the cancer without killing the patient, progress has been modest but significant improvements in survival did occur in premenopausal patients (2). Unfortunately, this is a hollow victory that on the face of it cannot be applied to cancer prevention; or can it?

We have known for more than a century that there is a link between the growth of breast cancer in patients and sex steroids secreted from the ovary (3) or produced peripherally in a woman's body fat. Furthermore, we have known for more than 30 years that combination cytotoxic chemotherapy will destroy ovarian function (reviewed in ref. 4) and stop estrogen production. Indeed, we now know that younger women who do not have a premature menopause and who do not take antiestrogen therapy have shorter survival than women who have ovarian failure (5-7). We also know that adjuvant oophorectomy produces disease-free survival comparable with the use of adjuvant cytotoxic chemotherapy in premenopausal women (8, 9). Thus, based on these clinical observations, one would be drawn to the conclusion that preventing hormone action might be a valuable line of future investigation for prevention if one could only work out the mechanism. But research does not travel in straight lines; a parallel universe of knowledge had already developed to address chemoprevention with antihormones.

An ovarian link between spontaneous breast (mammary) cancer in laboratory mice was shown in 1916 (10), but it was Professor Antoine Lassasagne (11) in 1936 who proposed that "a therapeutic antagonist should be sought to prevent the congestion of oestrone in the breast." In other words, an antiestrogen could be a valuable chemopreventive agent; however, at the time, there was no scientific foundation to support this strategy. The discovery of the estrogen receptor as the putative mechanism of estrogen action in its target tissues (12) opened the door to reinvent tamoxifen from a failed contraceptive (13) to become the first targeted therapy for breast cancer treatment (14). Tamoxifen, a nonsteroidal antiestrogen, was discovered in the 1960s as part of a worldwide effort by the pharmaceutical industry to exploit the serendipitous discovery of the drug group (15). Applications were sought based on *in vivo* studies and without reference to receptor mechanisms (16). The compounds were excellent postcoital contraceptive in rats but failed in this application because they induced ovulation in women (i.e., it could guarantee pregnancy), exactly the opposite effect that was being sought. As a result, tamoxifen was briefly marketed for the induction of ovulation (17). Although numerous compounds were discovered, only tamoxifen was reinvented as a long-term receptor targeted breast cancer treatment (ref. 18; and potential preventive ref. 19). A decade later, the drugs described as nonsteroidal antiestrogens (20) were recognized as selective estrogen receptor modulators (SERM) that could be estrogen-like at one site (i.e., bone or endometrial cancer) but antiestrogenic at another (i.e., breast; refs. 21-23). This discovery of SERM action (24) led to the proposition that it was plausible to prevent osteoporosis with SERMs in women but prevent breast cancer at the same time (15, 25). Raloxifene, a failed breast cancer drug (26), emerged as the first SERM used

to prevent osteoporosis with the beneficial side effect of preventing both breast and endometrial cancer (27-29). This was perfect timing as hormone replacement therapy used to prevent osteoporosis was shown to increase breast cancer incidence (30, 31).

The practical application of using tamoxifen for breast chemoprevention was pioneered by Trevor Powles (32, 33), Bernard Fisher (34, 35), and Umberto Veronesi (36, 37) who created a fundamental change in health care. There were no surprises as the ''good, the bad, and the ugly'' of laboratory research coupled with the vast resource of clinical experience with tamoxifen that reduced contralateral breast cancer when used as an adjuvant (38-40) were, in the main, predictive for the results in the chemoprevention trials. The ''good'' news was that tamoxifen reduced the risk of breast cancer in the large trials (34, 35, 41). Cuzick et al. (42) provided additional clinical trials data with the International Breast Intervention Study and did an ''overview analysis'' of all tamoxifen trials (plus the osteoporosis study with raloxifene; ref. 43). Tamoxifen is currently the only medicine that will reduce breast cancer risk safely and for prolonged periods (5 and probably 10 years) after therapy is stopped (35, 44, 45). This is remarkable and occurs at a time when there are no side effects. The advance with tamoxifen, now Food and Drug Administration approved for risk reduction in high-risk women for almost a decade, does have problems, but these seem to be overplayed by the media. Concerns about the ''bad'' side effects of endometrial cancer (generally good grade and curable) or blood clots and stroke are, in the main, associated with use in postmenopausal women. There is, however, a very small concern about uterine sarcomas (46, 47). Obviously, hysterectomized women are an appropriate target population for breast chemoprevention with tamoxifen.

The ''bad'' for some women is the increased incidence of menopausal symptoms. As it turns out, this may in fact be ''good.'' Tamoxifen needs to be metabolically activated to endoxifen by the *CYP2D6* gene product so patients with a variant *CYP2D6* usually have fewer hot flashes but have a higher recurrent rate (48, 49). Ironically, women who use the selective serotonin reuptake inhibitors paroxitene or fluoxetine to suppress hot flashes have a poor response to tamoxifen (48-50). This is because these selective serotonin reuptake inhibitors block tamoxifen metabolism. Venlafaxine is the selective serotonin reuptake inhibitor of choice because it does not block endoxifen production.

The ''ugly'' concern with tamoxifen was liver cancer induced in rats, but this did not translate to an increased incidence of hepatocellular carcinoma in women. It seemed to be obvious that this property, unique to rats, was not going to affect women, as the drug had already been marketed for 20 years at the time the hepatic toxicity was noted (51). No elevation in hepatocellular carcinoma are currently observed (8). Clinicians, however, do have another choice, raloxifene. This compound does not produce hepatocellular carcinomas in rats.

The SERM raloxifene had been rigorously investigated as a drug to prevent osteoporosis, and translational research predicted that this SERM would reduce the risk of breast cancer (21, 22, 25, 27). Based on this evaluation, the National Surgical Adjuvant Breast and Bowel Project chose to initiate the landmark SERM trial, the study of

tamoxifen and raloxifene or STAR (28). The results were clear and predictable: Tamoxifen and raloxifene were equivalent at reducing the risk of invasive breast cancer in high-risk women. There were trivial differences in ductal carcinoma *in situ* in favor of tamoxifen (probably due to the failure of compliance and the short duration of action of raloxifene when compared with tamoxifen; ref. 52) but the safety profile of the two SERMs favored raloxifene. Tamoxifen-treated women had more blood clots, more endometrial cancer, hysterectomies, and cataract operations compared with raloxifene-treated women. Supportive evidence for the value of raloxifene for the chemoprevention of breast cancer in postmenopausal women without a concern about an elevation of endometrial cancer comes from the trial named Raloxifene Use for the Heart (29). This trial was established to test the worth of raloxifene to prevent deaths from coronary heart disease but did not show an advantage for raloxifene over placebo. However, the trial showed a significant decrease in breast cancer and no elevation in endometrial cancer (29). Raloxifene is now a new weapon in the clinician's armamentarium to prevent breast cancer in osteoporotic women as well as postmenopausal women at high risk for breast cancer.

In closing, the question that needs to be addressed is why clinicians and women at high risk chose to avoid using approved medicines for appropriate indications? We have seen a dramatic change in the approach to breast cancer treatment and prevention in the past 30 years. Drugs can now be targeted to specific populations. In the case of prevention, tamoxifen is fully tested and is best used for high-risk premenopausal women with wild-type *CYP2D6* gene product, and venlafaxine can be used to control hot flashes. Raloxifene cannot be used in premenopausal women. Raloxifene is the agent of choice in postmenopausal women. Raloxifene is being used by an estimated 500,000 women to prevent osteoporosis, which will also prevent the development of tens of thousands of breast cancers over the next decade (53). The recent approval of raloxifene to prevent breast cancer in high-risk postmenopausal women will add to a reduction in breast cancer incidence while enhancing bone strength. The SERM concept (15, 25, 54, 55) works in medical practice and agents are available now to help the right patient. Only clinician and patient prejudice, convinced by negative media messages, is preventing progress in chemoprevention.

Returning to my original arguments about lung cancer, it is hard to believe that it is acceptable to smoke cigarettes with the attendant list of known health hazards and the highest death rate for cancer among women, but it is unacceptable to use approved medicines to reduce the risk of breast cancer. Fortunately, research is not static and new ideas will evolve and new SERMs will be developed, but, regrettably, progress will not occur in the near future. This is compounded by a lack of will by the government to support clinical research in chemoprevention and to support the training of a new generation of innovative clinical investigators. In the face of these obstacles, it is essential for the physicians to make the right choices for the appropriate patient. Interventions validated by decades of clinical and laboratory research and approved by the Food and Drug Administration can help reduce the risk of breast cancer now. After all, it's a once around life.

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# Estrogen Receptors in BRCA1-Mutant Breast Cancer: Now You See Them, Now You Don't

V. Craig Jordan

Estrogen receptor (ER) protein is expressed in estrogen target tissues (1,2). The binding of exogenous estrogen to ER orchestrates many important responses throughout a woman's body to maintain the optimal homeostasis for successful reproduction. Without estrogen, there would be no human race. However, estrogen is also involved in the development and growth of breast and endometrial cancers and, as a result, has recently earned a bad reputation in women's health (3,4).

The measurement of ER expression in breast tumors was originally used to identify which women were likely to respond to endocrine ablation therapy (5). Patients whose tumor expressed no ER were unlikely to respond to endocrine ablative surgery, whereas patients whose tumors had a detectable level of ER had improved chances of responding to ablative surgery (6). However, during the early 1970s ER was recognized as a therapeutic target for improving treatment rather than as a predictive test to recommend short-term palliation from endocrine ablative surgery (7,8). The antiestrogen tamoxifen was reinvented from being a failed contraceptive to the first targeted therapy in breast cancer (7,8). This conceptual shift led to the current recognition that the ER is perhaps the most important target identified thus far in cancer medicine. Hundreds of thousands of breast cancer patients' lives have been improved and lengthened with the application of long-term adjuvant tamoxifen therapy (9). Although the aromatase inhibitors are now improving response rates and the side-effect profile of long-term adjuvant therapy in postmenopausal women, tamoxifen remains the antiestrogenic treatment of choice for premenopausal women and those high-risk women who choose to reduce their chances of developing breast cancer (10).

Despite the prominence of the ER as a target in breast cancer, many aspects concerning its origins and its efficacy as a therapeutic target have remained a mystery. Questions about how ER synthesis and regulation are accomplished, whether ER-negative breast cancers are derived from ER-positive breast cancers, and whether ER expression can be regenerated in ER-negative breast cancers have remained central issues in endocrinology and cancer biology for the past 40 years.

In this issue of the Journal, Hosey et al. (11) provide a fascinating insight into these issues by presenting a unifying hypothesis for the regulation of ER synthesis in breast cancer. They approached these questions by integrating prior clinical observations that have shown that BRCA1-mutant breast cancers express little ER compared with spontaneous breast tumors (12) and then deployed breast cancer cell lines, nucleic acid transfection technology, chromatin precipitation assays, and, most importantly, the power of short-interfering RNA technology to knock down expression of BRCA1. They found that BRCA1 is a central player in the regulation of ER synthesis in breast cancer.

Overall, the current success by Hosey et al. (11) in answering the questions about ER regulation is best summarized by a statement taken from the book Trilobite! by Richard Fortey (13): "Central … is the notion of science as a web of knowledge where the apparently peripheral can suddenly become pivotal." Hosey et al. (11) have answered questions that could not have been answered 15 years ago. For example, the identification of the BRCA1 gene (14) and its mutations in familial breast cancer initially appeared to be unrelated to the ER, but the finding that breast tumors occur early during the premenopausal years of a woman's life and may have a hormonal component to their growth

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control (15,16) but, paradoxically, are ER-negative (12) provided a crucial piece of information necessary to solve the riddle of ER regulation. The question then became "what does a BRCA1 mutation have to do with the ER system?"

A connection between BRCA1 expression and ER has already been made by others. For example, Rosen's group (17,18) has demonstrated that the transient transfection of the wild-type BRCA1 gene into MCF-7 breast cancer cells inhibits signaling by the ER complex (17) and that BRCA1 protein interacts directly with ER (18). More recently, Rosen's group has shown that the repression of ER activity by BRCA1 is mediated through phosphatidylinositol-3 kinase signaling (19), which increases ER phosphorylation at serine 167 located in the activating function-1 domain of ER. All of these studies are interesting, but none directly addresses what a BRCA1 mutation has to do with the ER system.

Hosey et al. (11) took a direct approach to this question. They used three breast cancer cell lines: HCC1937 cells (20), which are homozygous for the BRCA1 5382insC mutation (which causes the last 34 amino acids of the BRCA1 protein to be missing) and are essentially ER negative, and the two ER-positive cell lines, MCF-7 (21) and T47D, which have different ER regulatory systems (22). Simply stated, Hosey et al. (11) showed that transfection of the wild-type BRCA1 gene into HCC1937 cells reactivates ER production and that the knockdown of BRCA1 expression with short-interfering RNAs in ER-positive cells eliminates expression of ER. They provide convincing evidence that BRCA1 protein directly regulates the synthesis of ER through binding to the ESR1 promoter and that the ubiquitous transcription factor Oct-1 also plays an important role in the regulation of ER expression. Finally, Hosey et al. (11) demonstrate that knockdown of BRCA1 expression in ER-positive cells abrogates the growth inhibitory response of the cells to the pure antiestrogen drug fulvestrant (23,24). They nicely show that expression of exogenous ER in BRCA1-depleted cells reactivates fulvestrant sensitivity. However, it would have been interesting to examine the effects of BRCA1 expression on the sensitivity of the cells to tamoxifen, a more clinically relevant antiestrogen drug. Fulvestrant is usually used as a second- or third-line antihormone therapy and is not really used to treat premenopausal patients, i.e., patients who tend to carry BRCA1 mutations. The fact that tamoxifen substantially enhances the development of mammary tumors in BRCA1 co/co MMTV-CRE/p53+/− mice and is more estrogen-like in cells with no full-length BRCA1 knockdown (25) suggests that this valuable observation should be pursued because of its clinical relevance.

Despite the large size of BRCA1, many mutations that alter the functions of the BRCA1 protein have been identified across the entire gene. The 5382insC mutation in the HCC1937 cells used by Hosey et al. (11), which is located in the terminal transactivation domain of BRCA1, and the 185delAG mutation are the two most common mutations found in the Ashkenazi Jewish population. Mutations for the BRCA1 gene occur with a combined frequency of about 100× higher in Ashkenazi Jews than in an unselected white population (26,27). Because 185delAG and 5382insC are the most severe mutations (i.e., they are associated with more aggressive, ER-negative breast cancers), the decision by Hosey et al. (11) to study a cell line that has the 5382insC mutation

was a wise one. However, it is possible that other mutations in the BRCA1 gene may explain why some BRCA1 mutant breast tumors remain ER positive and actually respond to tamoxifen treatment (16). This possibility would be interesting to test.

On the basis of their results, Hosey et al. (11) developed a plausible model to explain the formation of an ER-negative tumor through 1) the loss of ER expression after the wild-type BRCA1 allele is lost by a mechanism involving loss of heterozygosity and 2) the loss of BRCA1 expression in sporadic tumors by mechanisms involving loss of heterozygosity and epigenetic inactivation. Their model can now be rigorously investigated and validated so that the mystery of ER regulation can be settled once and for all.

In summary, the study by Hosey et al. (11) exemplifies the "notion of science as a web of knowledge where the apparently peripheral can suddenly become central" (13). The results of Hosey et al. (11) provide justifiable optimism that the current technology can be used to solve biologic questions. However, this is only one of the lessons to be learned from the advance made by Hosey et al. (11). The other lessons are that models are needed to solve mechanisms in biology and that there needs to be an integrated approach with different medical disciplines to address current research problems in biology and medicine. The discovery of mutations in the BRCA1 gene was clearly peripheral to the discovery of a plausible mechanism to explain the regulation of ER synthesis. The use of a breast cancer cell line (20) that was derived from a BRCA1 mutation carrier was critical for the demonstration that wild-type BRCA1 plays a role in ER synthesis. Perhaps most importantly, however, it is the financial investment in individual nondirected research that has provided the most powerful tools for investigators to solve problems. For example, Fire et al. (28) and Mello (29) studied the development of *Caenorhabditis elegans*, a transparent worm, and made the unanticipated discovery that a certain form of RNA would silence or interfere with the expression of genes. This discovery created and commercialized short-interfering RNAs for the whole human genome that ultimately allowed Hosey et al. (11) to silence genes selectively. They switched off ER synthesis by silencing the BRCA1 gene in two widely used ER-positive cell lines MCF-7 and T47D. Now you see the ER and now you don't. We do not live simply in interesting times; we live in exciting times.

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## Notes

Original article

# Exploiting the apoptotic actions of oestrogen to reverse antihormonal drug resistance in oestrogen receptor positive breast cancer patients

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## Abstract

The ubiquitous application of selective oestrogen receptor modulators (SERMs) and aromatase inhibitors for the treatment and prevention of breast cancer has created a significant advance in patient care. However, the consequence of prolonged treatment with antihormonal therapy is the development of drug resistance. Nevertheless, the systematic description of models of drug resistance to SERMs and aromatase inhibitors has resulted in the discovery of a vulnerability in tumour homeostasis that can be exploited to improve patient care. Drug resistance to antihormones evolves, so that eventually the cells change to create novel signal transduction pathways for enhanced oestrogen (GPR30 + OER) sensitivity, a reduction in progesterone receptor production and an increased metastatic potential. Most importantly, antihormone resistant breast cancer cells adapt with an ability to undergo apoptosis with low concentrations of oestrogen. The oestrogen destroys antihormone resistant cells and reactivates sensitivity to prolonged antihormonal therapy. We have initiated a major collaborative program of genomics and proteomics to use our laboratory models to map the mechanism of subcellular survival and apoptosis in breast cancer. The laboratory program is integrated with a clinical program that seeks to determine the minimum dose of oestrogen necessary to create objective responses in patients who have succeeded and failed two consecutive antihormonal therapies. Once our program is complete, the new knowledge will be available to translate to clinical care for the long term maintenance of patients on antihormone therapy.
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*Keywords:* Aromatase inhibitors; Tamoxifen; Raloxifene; Gene array analysis

## Introduction

The translation and application of long-term antihormonal strategies, aimed at the tumour oestrogen receptor (OER), has significantly improved the prognosis of patients with breast cancer.[1] Long-term adjuvant tamoxifen treatment not only enhances survival and disease-free survival in patients with OER positive tumours during treatment but also reduces mortality for at least 10 years after treatment has stopped.[2,3] Building on the success of long-term tamoxifen therapy, a number of aromatase inhibitors have been shown to improve prognosis and reduce side effects (blood clots and endometrial cancer) if given instead of tamoxifen[4–6] or after tamoxifen treatment.[7,8] Thus, the

original scientific strategy[9] of long-term antihormonal adjuvant therapy targeted to patients with OER positive disease[10,11] has emerged as the standard of care for breast cancer patients worldwide.

The new dimension of chemoprevention has advanced significantly during the past decade.[12] Preliminary studies were initiated in the 1980s to explore the safety and suitability of administering tamoxifen to women only at risk for breast cancer.[13–15] The rationale of these studies was based on the wide clinical experience using tamoxifen to treat all stages of breast cancer, the reduction of contralateral breast cancer noted in patients receiving adjuvant tamoxifen treatment[16–18] and laboratory studies that repeatedly demonstrated that tamoxifen can prevent mammary cancer in animal models.[19–22]

The current status and results of the worldwide efforts to quantitate and evaluate the value of tamoxifen as a

chemopreventive have been summarized recently[23] but it is the P-1 trial completed by Fisher and the National Surgical Adjuvant Breast and Bowel Project (NSABP)[24,25] that is considered to be the landmark.[26] The results can be summarized simply. Tamoxifen reduced the incidence of breast cancer by 50%[24] in pre and postmenopausal women at high risk.[27] Side effects noted were increases in early stage low grade endometrial cancer, blood clots, and cataracts[24,25] but only in postmenopausal women receiving long-term tamoxifen treatment. Tamoxifen is available in the United States for risk reduction in pre and postmenopausal women. However, the consensus today is that tamoxifen is better deployed as a chemopreventive for premenopausal women to reduce the risk of OER positive breast cancer.[28 32] There are no increases in the side effects of endometrial cancer or blood clots but tamoxifen keeps preventing breast cancer long after treatment stops[31] consistent with earlier treatment results.[3]

The concern that tamoxifen was going to be associated with the risk of endometrial cancer[33] and the recognition that the drugs called nonsteroidal antioestrogens[34] were in fact selective OER modulators (SERMs) led to a paradigm change for chemoprevention. SERMs were oestrogenic in ovariectomized rat bone[35] but at the same time prevented mammary cancer.[21] These data led to the evidence-based hypothesis that SERMs could prevent breast cancer as a beneficial side effect during the treatment and prevention of osteoporosis.[36,37] Based on this laboratory-based hypothesis, raloxifene was subsequently shown to reduce fractures in postmenopausal women with or at high risk for osteoporosis[38] but at the same time caused a 75% reduction in the incidence of breast cancer.[39] A follow-up trial P-2 by the NSABP[40] established that raloxifene was equivalent to tamoxifen at preventing invasive breast cancer in high risk postmenopausal women but with significantly fewer side effects (hysterectomies, cataracts, overall thrombolic events). However, although lower numbers of endometrial cancer were noted in raloxifene treated women compared to tamoxifen treated women, this was not significant because of a higher hysterectomy rate.[40] Nevertheless, a related trial called Raloxifene use for the Heart or RUTH, showed no increase in endometrial cancers during raloxifene treatment compared to placebo arm.[41]

Thus from this brief introduction, it can be appreciated that significant clinical advances have been made through the application of the principle of long-term antihormone therapy[9,36] for the treatment and prevention of breast cancer. All of the advances can now be applied in clinical practice to improve patient care. Nevertheless, despite these advances through the use of sustained administration of antihormonal drugs, there are consequences for the tumour with the eventual development of drug resistance. In the case of SERMs, the type of resistance is unique and is expressed as SERM stimulated growth.[42] But, it is the consistent study of the process of drug resistance to antihormones that resulted in the discovery[43] of a weakness in the mechanisms of antihormonal drug resistance that has potential for the future exploitation in clinical practice.

## Classification of SERM resistance

During the past 20 years we have focused our laboratory research program on developing models of SERM resistance in vivo to replicate events that could potentially occur clinically. The models were initially developed in vivo to avoid problems with cell culture where cells that become resistant to short term SERM treatment do not develop the essential requirements for angiogenesis that are necessary to survive and grow in patients. We now have a range of models that have been evaluated for growth in vivo (athymic mice) and that have been passaged in vivo for more than 5–10 years to replicate the long-term antihormonal therapy routinely used to treat patients (Table 1).

Initial studies of resistance to tamoxifen treatment demonstrated the unique feature of SERM stimulated growth. Resistant tumours that develop in athymic mice from both OER positive breast and endometrial cells grow in response to either a SERM or estradiol.[33,44] This is why an aromatase inhibitor or the pure antioestrogen fulvestrant (that binds to OER and facilitates the rapid destruction of the complex)[45] are successful second line therapies.[46,47] This form of resistance is referred to as Phase I resistance.[42]

However, these models represent only a few years of SERM treatment which is inconsistent with clinical experience of 5 years of adjuvant tamoxifen or possibly 10 years or more of raloxifene treatment to maintain bone density. The discovery that long-term SERM treatment exposes a vulnerability in the cancer cell that could have potential therapeutic applications was first reported at the St. Gallen meeting in the early 1990s.[43] Simply stated, long-term SERM treatment creates an absolute dependency on the SERM for tumour growth but small physiologic doses of oestradiol cause tumour cell death. Small tumours respond more readily to the apoptotic action of oestrogen but when tumours regrow during continuous oestrogen

Table 1
The available SERM resistant OER positive tumours used to investigate drug resistance in our laboratory.

| Phase | Organ site | SERM | Cell line | Reference |
|---|---|---|---|---|
| I | Breast | tamoxifen | MCF 7 | 44, 67, 68 |
| | Breast | tamoxifen | T47D | 69 |
| | Endometrial | tamoxifen | human tumour | 33 |
| | Endometrial | tamoxifen | ECC 1 | 70 |
| II | Breast | tamoxifen | MCF 7 | 43, 48, 71 |
| | Breast | raloxifene | MCF 7 | 72 |
| | Endometrial | raloxifene | ECC 1 | (unpublished) |

Phase I resistance refers to tumours that can be stimulated to grow into oestrogen or a SERM whereas Phase II resistance refers to tumours stimulated to grow only with a SERM. Oestrogen causes Phase II tumors to undergo apoptosis and regress.[42]

therapy, the tumours again respond to the SERM or no treatment[48] (equivalent to treatment with an aromatase inhibitor for patients). This form of resistance is referred to as Phase II resistance.[42] The models for SERM resistance are summarized in Table 1. Thus, it is plausible to consider a clinical strategy whereby limited duration, low dose oestrogen treatment could be used to purge and destroy Phase II resistant breast cancer cells but then patients could be treated again with antihormonal therapy to control tumour growth. However, a case could be made that the ubiquitous use of tamoxifen is declining and over the next decade the standard of care will be long-term treatment with one of several aromatase inhibitors. The question we have addressed in the laboratory is whether long-term oestrogen deprivation of breast cancer cells will expose the vulnerability to the apoptotic actions of oestrogen.

Table 2
The basic characteristics of the MCF 7 cell lines developed from long term oestrogen deprivation.

| | Cell Line | |
|---|---|---|
| | MCF 7:2A | MCF 7:5C |
| OER | ++ | ++ |
| Oestrogen induced PgR | ++ | |
| GPR30 | ++++ | ++++ |
| Growth inhibitory response to SERMs | ++ | |
| Growth inhibitory response to fulvestrant | +++ | ++ |
| Invasion proteins | ++ | ++++ |

Results are replicate (5) data from the affymetrix U 133 gene arrays relative to wild type MCF 7 cells. However, the biology of responses to antioestrogens are based on cell growth experiments where no effect is and 100% response is ++++.

## Resistance of breast cancer to oestrogen deprivation

There are two laboratory approaches to developing models of drug resistance to aromatase inhibitors. The traditional model is to study the impact of oestrogen withdrawal on the growth of OER positive breast cancer cells. In contrast, there is a model in vivo employing athymic mice transplanted with MCF-7 cells stably transfected with the aromatase enzyme. Without oestrogen tumours do not grow but when animals are treated with the enzyme substrate androstenedione to make oestrogen, tumour growth occurs. Simultaneous treatment with a number of aromatase inhibitors results in initial control of oestrogen-stimulated tumour growth but then the inhibitors fail and tumour growth occurs despite continuing treatment. This approach has been most instructive about strategies for antihormonal sequencing and the rationale of avoiding a combination of a SERM and an aromatase inhibitor for breast cancer therapy.[49,50]

The traditional approach of oestrogen withdrawal using breast cancer cells not engineered in any way, was not possible until Berthois and coworkers[51] discovered that cell culture media contained significant quantities of oestrogen found to increase the growth rate of MCF-7 cells. In other words, despite the fact that investigators were adding charcoal stripped serum to remove endogenous oestrogen, the media already contained oestrogenic chemical contaminants from the phenol red pH indicator.

Initial studies of the short and long-term effects of oestrogen deprivation of MCF-7[52,53] and T47D[54] breast cancer cells noted some interesting differences based on the regulation of OER in the different cell types.[55] The MCF-7 cells that are obtained following long-term oestrogen
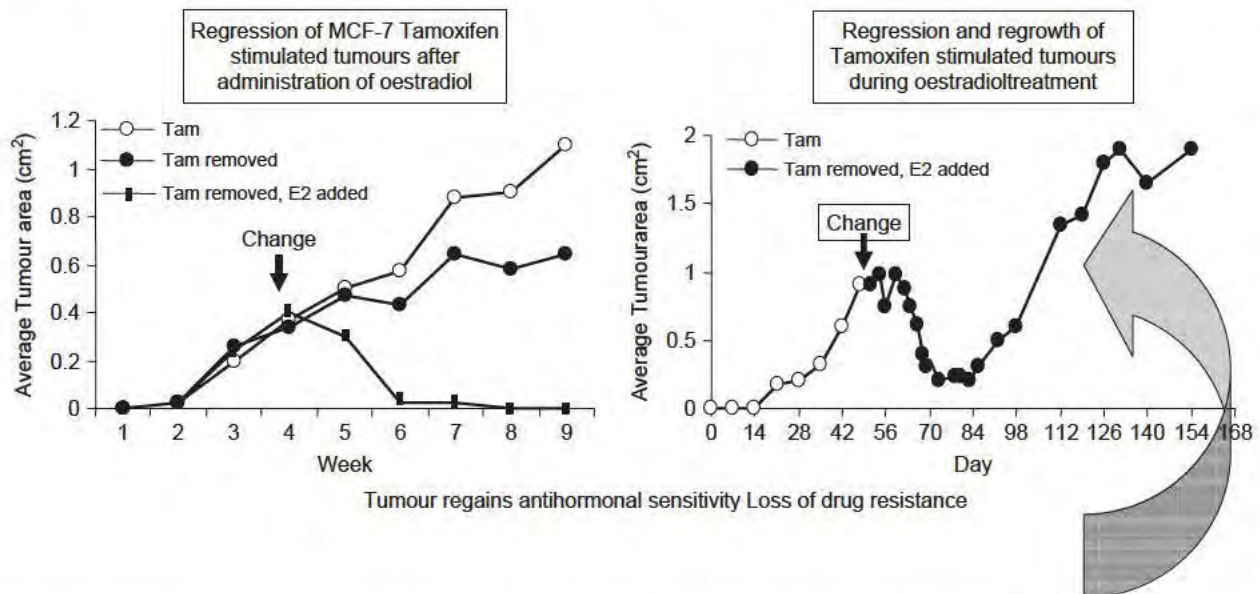


Fig. 1. Diagrammatic representation of the actions of physiologic oestradiol (E2) on the growth of small phase II MCF 7 tamoxifen resistant tumors in ovariectomized athymic mice. A larger tumour will regress with oestrodiol treatment but will eventually display oestrogen stimulated growth. If tumours are retransplanted into a new generation of ovariectomized athymic mice and treated with oestradiol, tamoxifen will block oestrogen stimulated tumour growth.[48] First presented in St. Gallen, 1993.[43]

deprivation remain OER positive (Table 2) whereas the T47D lose the OER.[56] The levels of OER increase in the oestrogen deprived MCF-7 cells (Table 2) and also there are increases in GPR30[57] noted in our gene array data. Thus, the oestrogen-deprived cells have an enhanced signal transduction pathway to support survival. Since breast cancers seem to rarely lose the OER efforts to study antihormonal drug resistance have focused on the MCF-7 line.

Our program to develop MCF-7 cell lines resistant to oestrogen withdrawal successfully described two clones of cells: the MCF-7:5C and the MCF-7:2A line. The MCF-7:5C line[58] is OER positive but progesterone receptor (PgR) negative and unresponsive to both oestrogen and SERM treatment. In contrast, the MCF-7:2A cell line[59] did respond to SERM therapy with a reduction in growth rate but oestrogen did not affect the growth rate, except at high concentrations. We have known for nearly 20 years that activation of growth factor receptor pathways can create intrinsic SERM resistance[60,61] and a down regulation of PgR induction.[62] These data would be consistent with the finding for the MCF-7:5C cells (Table 2). The laboratory observation that deactivation of the OER signal transduction pathway with fulvestrant is consistent with clinical observation that fulvestrant produces reasonable control of aromatase resistant breast cancer.[63] However, the models of oestrogen deprivation we developed in the early 1990s were to take center stage once the SERM resistant models were found to be reproducible[48] and worthy of further development (Table 1). The key to the value of the two MCF-7 clones (5C, 2A) was that they could be studied in vitro to understand the mechanism of oestrogen-induced apoptosis using genomics.

## The new biology of oestrogen action

A re-examination of MCF-7 clones 5C and 2A occurred at the time when clinical investigators were re-examining the value of high dose oestrogen therapy in those patients who had been treated exhaustively with successive antihormonal therapies.[64] The clinical studies demonstrated that high dose oestrogen therapy could cause tumour regression or stasis (30%) in patients treated exhaustively with antihormones.[64] Additionally, high concentrations of oestrogen could induce apoptosis in long-term oestrogen deprived cells in culture.[65] In contrast, we pursued our original hypothesis that the apoptotic supersensitization of breast cancer cells by long-term antihormonal therapy could occur with physiologic or a very low concentration of oestrogen treatment.[43,48]
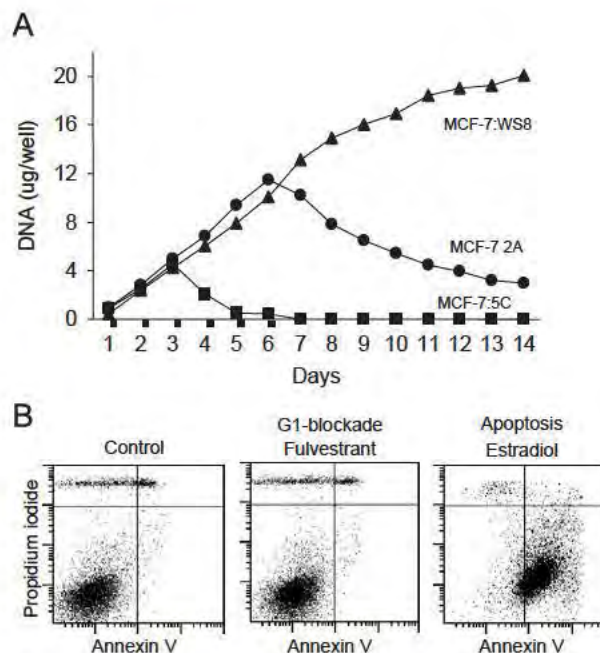


Fig. 3. The action of oestradiol (1 nM) on the growth of wild type MCF 7 cells (WS8) or long term oestrogen deprived MCF 7 cells (5C and 2A). In Panel A the MCF 7:5C cells undergo rapid apoptosis during the first few days of oestradiol exposure whereas the MCF 7:2A cells slowly initiate apoptosis during the days after 6 of oestradiol treatment. In panel B MCF07:5C cells respond to fulvestrant (1 μM) with a G1 blockade at 72h whereas oestradiol (1 nM) causes massive and complete apoptosis. These results were obtained using flow cytometry.
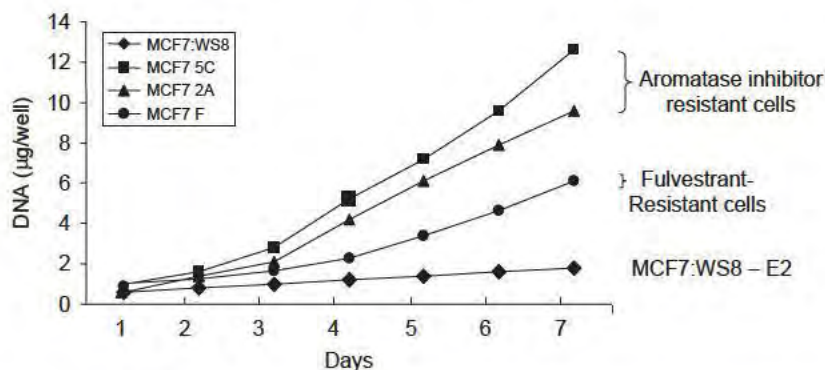


Fig. 2. The growth of wild type MCF 7 cells (WS8) and various antihormonally resistant sublines in an oestrogen free environment. The cells MCF 7:5C and 2A grow spontaneously and could be considered to represent aromatase inhibitor resistant cells. These remain OER positive. In contract, MCF 7F are fulvestrant resistant (MCF 7 cells grown for over a year in an oestrogen deprived environment containing fulvestrant). These cells grow spontaneously but have no OER.
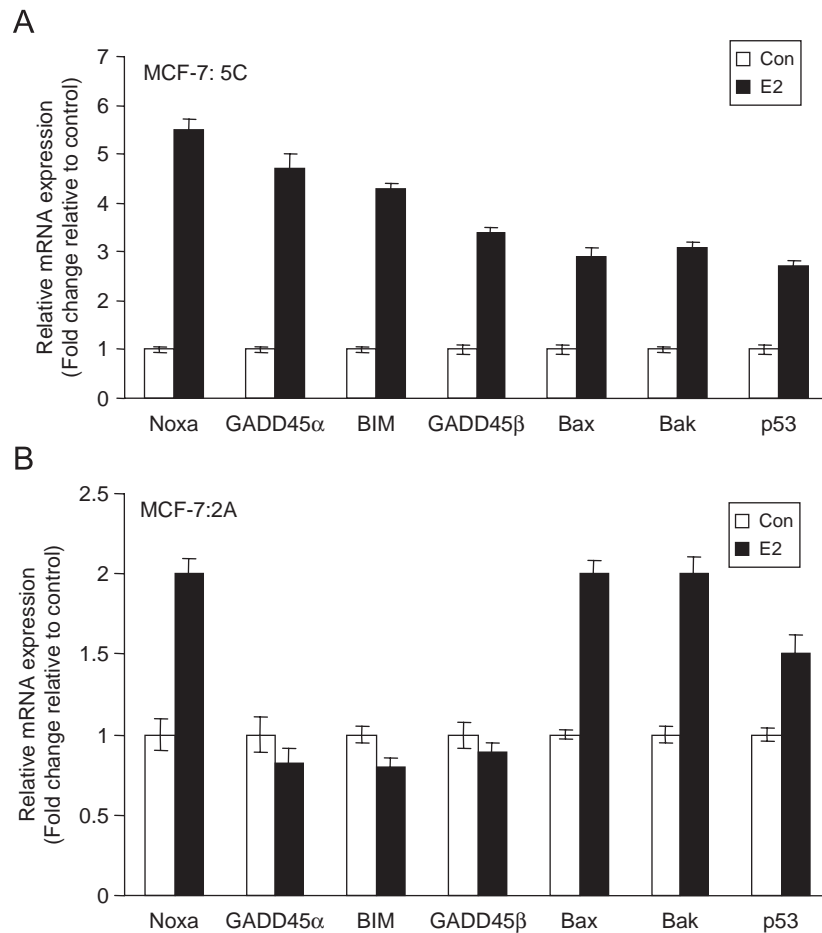
Fig. 4. Oestrogenic regulation of apoptotic genes in long term estrogen deprived MCF 7:5C and MCF 7:2A breast cancer cells as determined by Affymetrix gene microarrays. For experiment, cells were treated with 1 nM oestradiol for 48 h and total RNA was prepared using the Qiagen Rneasy Mini kit. cRNA was generated, labeled, and hybridized to the Affymetrix Human Genome U133 plus 2l0 arrays containing 54,300 probe sets. Chips were then scanned and analysed using the Affymetrix Microarray Analysis Suite version 5.0. Assessment of data quality was conducted following default guidelines in the Affymetrix's GeneChip® Expression Analysis Data Analysis Fundamentals Training Manual. Global scaling for average signal intensity for all arrays was set to 500. Four biological replicates from each of the two cell lines were arrayed to determine consistent and reproducible patterns of gene expression. The above figure shows that oestradiol treatment caused 3 to 6 fold induction of the proapoptotic genes NOXA, GADD45α, GADD45β, BIM, BAX, BAK and p53 in (A) MCF 7:5C cells but only a 2 fold induction of NOXA, BAX, and BAK in (B) MCF 7:2A cells.

Two important observations, that were made during the re-evaluation of the MCF-7:5C and 2A cells, reinforced the view that oestrogen-induced apoptosis could be applied to reverse resistance to aromatase inhibitors. The first observation occurred by changing the charcoal stripped serum from the original 5% charcoal stripped calf serum[58] to 10% developed stripped fetal bovine serum.[66] This caused a dramatic increase in the growth rate of the 5C cells to be comparable to the MCF-7:2A cells (Figs. 1 and 2). Remarkably, physiologic oestradiol (lnM) now caused a massive apoptotic response in the MCF-7:5C cells (Fig. 3A,B). The MCF-7:2A cells had previously[59] been found to be responsive to antioestrogens by inhibiting growth and oestrogen by inducing progesterone receptor synthesis. The 2A cells, however, only weakly responded to the growth inhibitory effects of high concentrations 1 μM oestradiol. This original assumption is not true if the time course is extended (Fig. 3A). The 2A cells appear to have a survival mechanism that is able to protect them initially

from the apoptotic actions of oestradiol. Nevertheless, this survival mechanism eventually fails. Overall, our models now create an interesting opportunity to interrogate the time courses with genomics and proteomics to find the precise oestrogen-induced mechanisms for protecting the cell from apoptosis.

**Analysis of apoptotic pathways**

A number of U-133 Affymetrix gene arrays were completed using the MCF-7, MCF-7:5C and 2A cell lines to define the early events of oestrogen action. A 48 h time point was used in our preliminary studies and five replicates were analysed to ensure statistical veracity. All gene array analyses were completed at Translational Genomics, AZ. Results illustrated in Fig. 4 show the 48 h increase in proapoptotic genes that are activated by oestrogen in the MCF-7:5C cells. This is consistent with the time course for the apoptotic death response of the
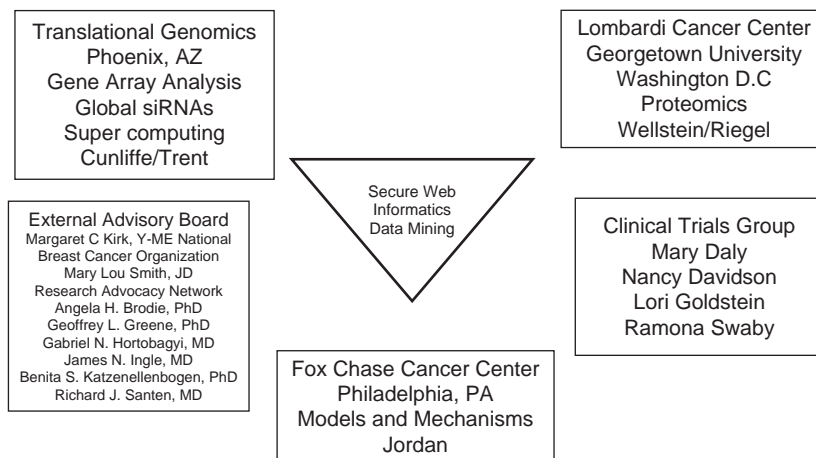
Fig. 5. The organization of our Department of Defence Center of Excellence Grant entitled "A New Therapeutic Paradigm for Breast Cancer Exploiting Low Dose Estrogen Induced Apoptosis." The model systems to study the survival and apoptosis induced with oestrogen are used for time course experiments at the Fox Chase Cancer Center. The materials are distributed to Translational Genomics for siRNA analysis or gene array and the Vincent T. Lombardi Cancer Center is involved to conduct proteomics. All results are uploaded into a shared secure web for data processing and target identification by our informatics and biostatistical group. Each laboratory is able to validate emerging pathways and study individual genes of interest. Our program is integrated with a clinical trials program that provides patient samples for validation of apoptotic or survival pathways. We are grateful to our external advisory board of Patient Advocates and professional colleagues for their continuing advice and support.

MCF-7:5C cells noted in Fig. 3. In contrast, oestrogen had not yet activated the full apoptotic response in MCF-7:2A cells that become apoptotic over a much longer time course (Fig. 3).

Overall, we have confirmed our novel observations that breast cancer and endometrial cancer cells (unpublished observation) become resistant to long-term antihormonal interventions by reconfiguring the oestrogen signal transduction pathway to induce an apoptotic response rather than enhancing survival and further growth. These data plus the emerging anecdotal results of clinical case reports (James Ingle, MD and Mr. Michael Dixon personal communications) prompted us to develop a multicenter program to explore our unique model systems systematically so that we can describe the mechanisms of oestrogen-induced survival and apoptosis in breast cancer. Completion of these studies would then provide an invaluable database to translate to patient care. The goal would be to determine the lowest dose of oestrogen necessary to cause apoptosis in a significant number of women whose tumours no longer respond to antihormonal therapy. This would reverse antihormone resistance in a significant proportion of patients.

**Translation of laboratory results to patient care**

We have established a multi-center collaborative translational research grant with headquarters at the Fox Chase Cancer Center (FCCC) (Figs. 5 and 6). The five year program is sponsored by the US Department of Defense Breast Cancer Program BC050277 entitled "A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis."

Our goal is to create maps of the survival and apoptotic responses to oestrogen noted in our models in vivo and in vitro. Biological samples from our time course experiments using our models at the FCCC are being distributed to Translational Genomics in Arizona for Agilent gene array analysis, CGH and CpG methylation arrays. Total human genome siRNA analysis is also being completed on our cell lines. Additionally, samples for proteomics are being dispatched to Georgetown University (Vincent T. Lombardi Cancer Center, PIs Anton Wellstein and Anna T. Riegel). All processed data are then being uploaded into a secure website for data mining and target identification, so that verification and validation studies can occur at each of the collaborating sites. A clinical program is exploring the clinical applications of our laboratory observation with two successive protocols:

(1) A single arm phase II study of pharmacologic dose oestrogen in postmenopausal women with hormone receptor-positive metastatic breast cancer after failure of sequential endocrine therapies.
(2) Reversal of anti-estrogen resistance with sequential dose de-escalation of pharmacologic oestrogen in a single arm phase II study of postmenopausal women with hormone receptor-positive metastatic breast cancer after failure of sequential endocrine therapies.

Our clinical studies are in place (1) to confirm the clinical finding[64] that high dose oestrogen treatment following exhaustive antihormonal treatment of OER positive breast cancer will give a 30% response rate and (2) to determine the lowest dose of oestrogen that will induce an equivalent tumour regression as high dose oestrogen (30 mg. oestradiol daily). All patients will be monitored weekly using the Apoptosense® serum assay to detect apoptotic markers in responding and non-responding patients. Additionally, where possible, patients will have biopsies of accessible

## TREATMENT PLAN FOR THIRD LINE THERAPY
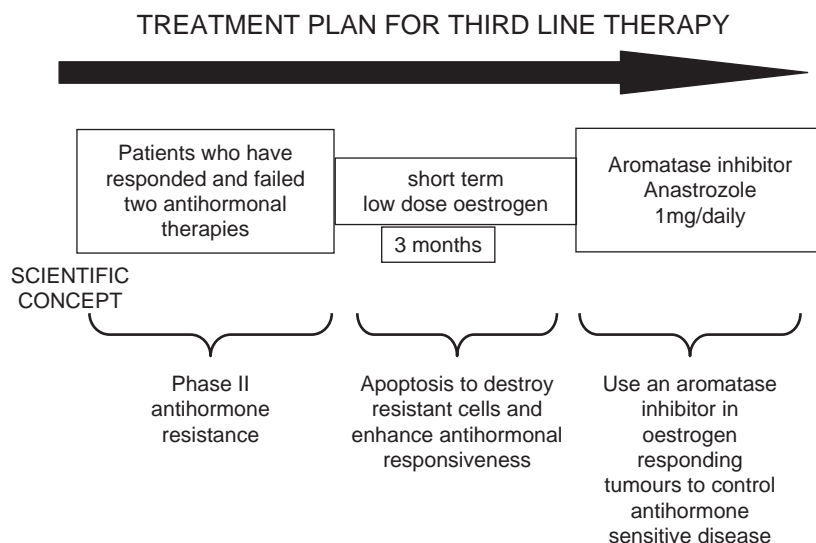


Fig. 6. An anticipated treatment plan for third line endocrine therapy. Patients must have responded and failed two successive antihormonal therapies to be eligible for a course of low dose oestradiol therapy for 3 months. The anticipated response rate is 30%[64] and responding patients will be treated with anastrozole until relapse. Validation of the treatment plan via the Center of Excellence grant (Fig. 5) will establish a platform to enhance response rates with apoptotic oestrogen by integrating known inhibitors of tumour survival pathways into the 3 month debulking treatment plan. The overall goal is to increase response rates and maintain patients for longer on antihormonal strategies before chemotherapy is required.

tumour tissue before and after 12 weeks of oestrogen therapy (or shorter if patients rapidly progress). Responding patients will be retreated with 1 mg anastrozole daily until progression.

Overall, the map of survival and apoptotic pathways we create from our laboratory models will be invaluable to guide our selection of target genes in biopsies using real time RTPCR. This will provide clues as to our future strategy of improving response rates with agents that selectively block survival pathways which can then be used in combination with our apoptotic oestrogen purge. It is our long term goal to improve oestrogen-induced response rates in patients refractory to antihormonal therapies. In so doing, select patients with metastatic breast cancer can anticipate longer disease control before chemotherapy is necessary. Most importantly, the new knowledge will provide an in silico platform to identify the apoptotic target so effectively located by the OER.

## Conflict of Interest

None declared.

## Acknowledgements

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# Selective Estrogen-Receptor Modulators and Antihormonal Resistance in Breast Cancer

*V. Craig Jordan and Bert W. O'Malley*

## ABSTRACT

Selective estrogen-receptor (ER) modulators (SERMs) are synthetic nonsteroidal compounds that switch on and switch off target sites throughout the body. Tamoxifen, the pioneering SERM, blocks estrogen action by binding to the ER in breast cancers. Tamoxifen has been used ubiquitously in clinical practice during the last 30 years for the treatment of breast cancer and is currently available to reduce the risk of breast cancer in high-risk women. Raloxifene maintains bone density (estrogen-like effect) in postmenopausal osteoporotic women, but at the same time reduces the incidence of breast cancer in both high- and low-risk (osteoporotic) postmenopausal women. Unlike tamoxifen, raloxifene does not increase the incidence of endometrial cancer. Clearly, the simple ER model of estrogen action can no longer be used to explain SERM action at different sites around the body. Instead, a new model has evolved on the basis of the discovery of protein partners that modulate estrogen action at distinct target sites. Coactivators are the principal players that assemble a complex of functional proteins around the ligand ER complex to initiate transcription of a target gene at its promoter site. A promiscuous SERM ER complex creates a stimulatory signal in growth factor receptor–rich breast or endometrial cancer cells. These events cause drug-resistant, SERM-stimulated growth. The sometimes surprising pharmacology of SERMs has resulted in a growing interest in the development of new selective medicines for other members of the nuclear receptor superfamily. This will allow the precise treatment of diseases that was previously considered impossible.

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## INTRODUCTION

The estrogen receptor (ER) is the trigger[1] that initiates estrogen action in its target tissues (eg, uterus, vagina, and pituitary gland). The subsequent identification of the ER in some breast cancers created a mechanistic link to explain the hormonal dependence of some breast cancers.[2] Ultimately, this knowledge was used to reinvent a failed postcoital contraceptive, ICI 46474,[3] as tamoxifen, the first targeted antiestrogenic therapy for breast cancer.[4] The clinical strategy of targeting ER-positive breast tumors with long-term adjuvant therapy has saved hundreds of thousands of lives.[5] As a result, the evolving use of tamoxifen therapy during the last three decades has proved to be the cornerstone for the treatment and prevention of breast cancer.[6]

However, the recognition[7] that the "nonsteroidal antiestrogens" were, in fact, selective estrogens and antiestrogens at different target tissues around the body, created a new dimension in drug development and enhanced therapeutic possibilities. The selective estrogenic properties of tamoxifen and raloxifene maintained bone density[8] but the selective antiestrogenic properties prevented rat mammary carcinogenesis.[9] These laboratory data were used to develop an evidence-based therapeutic strategy[10,11] that has now become a clinical reality with the development of raloxifene. This second-generation selective ER modulator (SERM) prevents osteoporosis but also prevents breast cancer as a beneficial side effect.[12] With this significant advance in therapeutics, it has become clear that the action of SERMs at different target sites can no longer be explained by an ER model that simply turns estrogen action on or off. Other physiologic factors must be involved.

In this article, we will describe our evolving understanding of SERM action at its target sites. Although the ER complex is programmed by the shape of the SERM buried inside the receptor, it is the new protein players called coactivators and corepressors[13] that are now known to modulate and control the dynamics of the complex as it turns on or turns off subcellular signaling networks at target sites around the body. However, we believe it is important to state at the outset that although we have, by necessity, chosen to explain the molecular mechanism of SERMs to retain therapeutic relevance in oncology, we prefer to use the term steroid

receptor modulators (SRMs) when considering mechanisms. The molecular biology of selective activity is clearly universal within the steroid receptor superfamily.[13] This fact has important therapeutic implications for future drug discovery.

## MECHANISMS OF SELECTIVE RECEPTOR MODULATOR ACTION

Of the 48 members of the nuclear receptor (NR) family, approximately half have been determined to be regulatable by ligands.[14,15] The remaining molecules are regulated by signaling pathways that impart post-translational modifications to these endocrine/metabolic transcription factors. The nuclear receptors are signal-dependent transcription factors that have two main purposes: (1) to locate target genes by binding at specific DNA sequences (termed hormone response elements [HREs]) that are located at these genes; and then, (2) to recruit transcriptional coregulators to the gene.[16] Ligands can induce both activation and repression of target genes. NRs recruit coactivators to activate genes, and corepressors to repress genes.[17,18] These two functionally different classes of molecules comprise the totality of 285-member coregulator superfamily, most of which are coactivators. The general

domain structure of coactivators is shown schematically in Figure 1, and a great deal of additional basic and clinical information is provided on the Nuclear Receptor Signaling Atlas Web site (www.nursa.org). Although the NR coregulators were identified only approximately 11 years ago,[19] they are generally accepted as the rate-limiting components of transcriptional control in mammals.

The molecular mechanisms by which distinct ligands can bind to the same nuclear receptor and yet exert tissue-specific actions, has been somewhat of a mystery until the last decade, when the contributions of basic receptor research have led to an enlightened viewpoint.[13] We now realize the complexities and the relative importance of the fundamental elements that factor into the equations for tissue-selective SRM actions. These elements are (1) receptor isoform subtypes; (2) ligand-induced conformations of the receptor; (3) precise sequence compositions of the HREs; (4) nuclear receptor coregulators (coactivators and corepressors), which are recruited by the active or inactive conformation of the receptor to the gene site; and (5) cell and signaling context. Although the coregulator recruitment is of paramount importance, under most conditions, all five of the preceding events can have a modulating influence on the actions of an SRM.
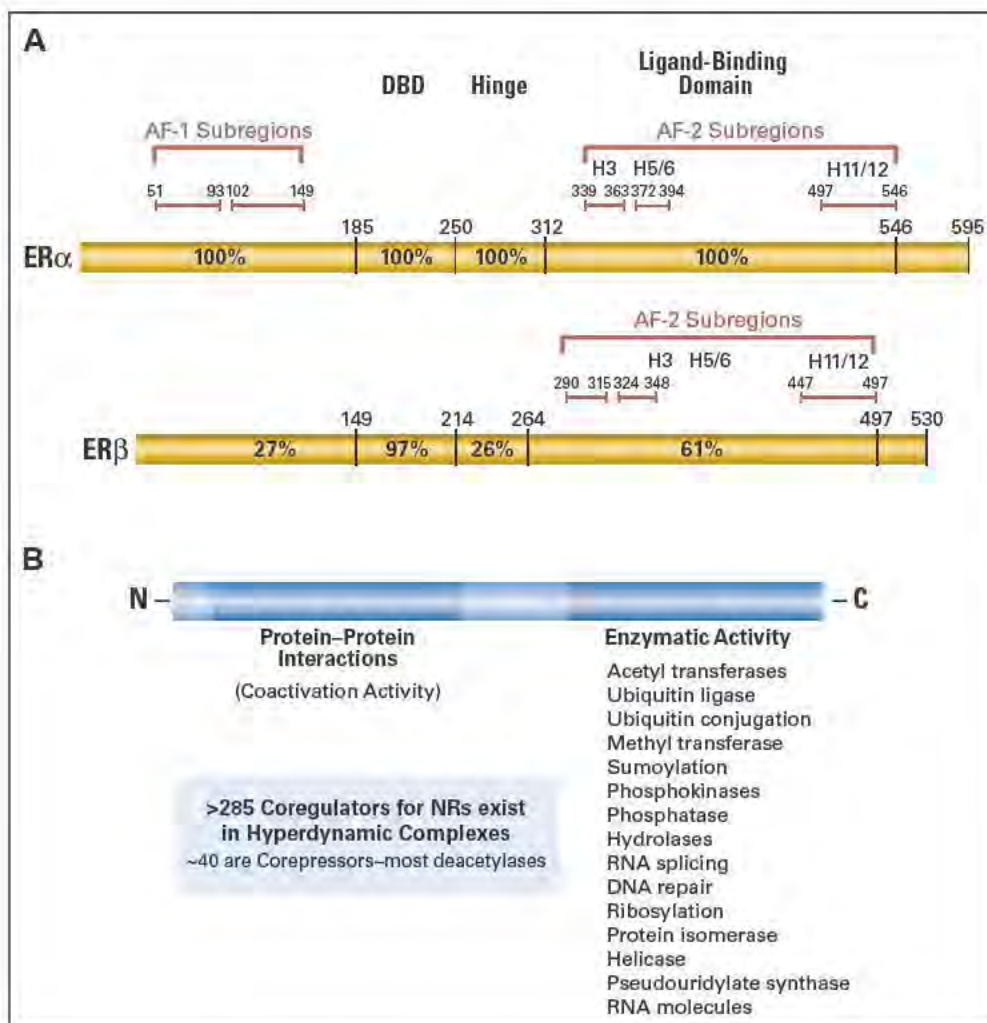


Fig 1. (A) The structure of the known estrogen receptors (ERs) with the identified (red) activating functions (AFs) that bind coactivators. Also identified is the DNA-binding domain (DBD) and the ligand binding domain. (B) A typical domain structure of a nuclear receptor (NR) coactivator is shown. There are two main domains: (1) a protein-protein interacting domain that binds other cocoactivators in the functional high molecular weight coactivator complex and (2) an enzymatic domain that either has intrinsic enzyme activity or binds a protein that has enzyme activity. Numerous enzyme activities have been demonstrated in the many coactivators discovered to date.

## RECEPTOR ISOFORMS

Multiple function-specific isoforms have been discovered for a number of receptors, including those for progesterone receptor (PR; PRa, PRb), ER (ERα, ERβ), and glucocorticoid receptor (GR; GRα, GRβ).[15] These isoforms have different primary structures and therefore beget different gene functions. Since the tissue concentration of receptor isoforms can vary in a tissue-specific manner, the functions of the cognate receptor ligand in a given tissue can vary also. Perhaps the ERα and the ERβ isoforms have the most contradictory functions, with ERα having a growth promoting action and ERβ having a growth-inhibitory action in certain tissues.[20] Consequently, the tissue-selective ratio of ERα/ERβ can provide a tissue-selective function.

## LIGAND-INDUCED RECEPTOR CONFORMATION

For many years it was suspected that a transcription-inducing ligand acted simply by shifting the equilibrium of its cognate NR from an inactive to an active conformation. Two complimentary experimental approaches helped to clarify receptor-mediated modulations. A comprehensive pharmacologic evaluation of the structure function relationship of estrogens and antiestrogens both at an ER-regulated prolactin gene target[21,22] and by regulating breast cancer cell replication,[23] built up a hypothetical model of molecular modulation. The pharmacologic studies concluded the size and position of the "antiestrogenic" side chain of the then nonsteroidal antiestrogens controlled the folding of the ER at an antiestrogenic region of the ER.[21,24,25] Simply stated, the "crocodile" model proposed equilibrium mixtures of receptor jaws closed (estrogenic complex) or propped open by the ligand (partial estrogenic/antiestrogenic complex) to modulate gene function at target sites.[26,27] Complementary early biochemical studies utilized protease structural mapping and antibody epitope mapping techniques to demonstrate that progesterone and estrogen bound to their cognate receptors and induced a conformational alteration in the carboxy-terminal tail of the receptor, whereby the tail flipped back over the ligand pocket and the active form was stabilized.[28,29] It was the eventual x-ray crystallography of these molecules, however, that provided a more detailed picture of this model, whereby a c-terminal helix 12 was the lid that covered the ligand pocket and formed a landing platform for newly recruited coactivators (or corepressors).[30-32] The newly recruited coregulators then carry out all of the reactions required for the entire transcriptional process (discussed further herein). Different receptors binding to the same genetic sequence can recruit different coactivators and thereby provide quantitatively or qualitatively different gene responses (Fig 2). Similarly, different ligands occupying the same receptor at a gene site can induce different structural conformations in that receptor and lead to recruitment of different coactivators, and consequently, different gene expression patterns.

## DNA BINDING ELEMENT (HRE) OF THE TARGET GENE

The precise composition of different genomic HREs in mammals varies. HREs are usually composed of short inverted or direct repeats of approximately 7 deoxynucleotides each. When minor variations in the receptor contact sequence occur, and in combination with other surrounding transcription factors, the receptor can be forced into an altered conformation that in turn recruits different coregulators and provides distinct functions for these genes, if they are expressed in that tissue.[33] This basic principle has been demonstrated, but it is unclear as to how often this is a significant factor in SRM actions. What is clear is that recruitment of the receptor complex to the HRE is cyclical with binding and destruction.[34]

## NUCLEAR RECEPTOR COREGULATORS

Current opinions place the coregulators in the driving seat of tissue-specific actions of SRMs. The potency and selectivity for all subreactions of transcription reside in these coregulators, and thus, they are critically important for not only gene function, but also tissue-selective gene function. Currently there are approximately
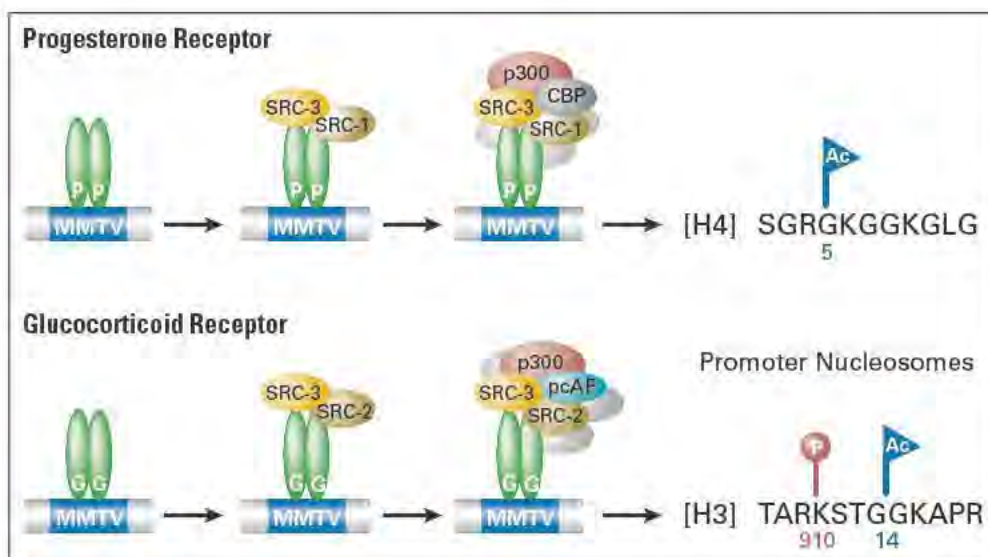


Fig 2. Differential recruitment of coactivators to same gene by receptors determine specific promoter chromatin modifications and transcription initiations. The top panel contains a schematic of the progesterone receptor bound to the hormone response element (HRE) of the *MMTV* gene. The bottom panel demonstrates that when the glucocorticoid receptor is bound to the same HRE of the *MMTV*, it accumulates different coactivator proteins. Each of the receptors induce different patterns of histone modifications and subsequent transcription. The panels illustrate that the ligand-bound receptor itself plays the dominant role in what coactivators are recruited, and thus can modify target gene transcription accordingly. Ac, acetylation; P, phosphorylation.

285 NR coregulators, of which the vast majority are coactivators (approximately 40 are corepressor according to the Nuclear Receptor Signaling Atlas). Most occur in the majority of tissues, but at different individual concentrations in each tissue. Consequently, each tissue has a "quantitative finger print" of coactivators based on the relative concentrations of each molecule in that tissue.[16] This inherited complement of coregulators provides a basis for tissue-selective actions by a given NR.

Coregulators function as large, high-molecular weight complexes of approximately six to seven cocoregulator proteins.[17] Most of the coregulators are enzymes that participate in remodeling the local chromatin structure at the target promoter, initiating transcription by RNA polymerase, encouraging efficient elongation of RNA chain synthesis, regulating alternative RNA splicing, and, finally, destroying the active transcription factors at the promoter site. These series of substeps of transcription occur in rapid sequence (approximately 15 seconds apart) and are controlled by sequential occupation of the promoter by specific coregulator complexes that direct the transcriptional substep reactions.

For the most part, the coregulators are themselves regulated at the post-transcriptional level.[17] Their intracellular concentrations are determined by their proteasomal degradation rates. Levels are raised by inhibiting the rate of degradation, and vice versa for lowering levels. Traditional ubiquitin-mediated degradation occurs, as well as an ubiquitin-independent turnover by 11S cap proteins such as REGγ.[35] Degradation can be inhibited by post-translational modification of a coactivator at certain sites; alternatively, specific kinases can phosphorylate these sites to promote higher cellular levels of coactivator.

## CELL AND SIGNALING CONTEXT

The cell context plays a role in selective gene responses to ligand because differentiation produces cells with specific available gene complements for expression. The cell also has a predetermined basal concentration of each of the coregulators and their cognate activating/inactivating enzymes, thereby establishing a threshold of available regulatory molecules. This cellular concentration of coregulators provides the potential for activity. For actual conversion to active functional molecules, however, the coregulators must be regulated by a variety of post-translational modifications, such as phosphorylation, ubiquitinylation, acetylation, SUMOylation, methylation, etc. In general, coactivators are activated by phosphorylations and mono-ubiquitylations.[36] Protein-protein interactions in the large coactivator complexes are regulated by acetylations and methylations. Coactivators are inactivated by SUMOylation and degraded after poly-ubiquitylations. These general rules often vary for a given coactivator. Considering the crucial role that post-translational modifications play in coactivator function, it is logical to assume that the roles of signaling pathways that contain these modifying enzymes also play important roles. Since the signaling pathways have certain cell specificities and are subject to environmental stimuli for their regulation, cell context can play a role in selective activities of SRMs.

## OTHER REGULATORY INFLUENCES

Because equilibrium reactions are the basis for biology, the promotional and contradictory influences inherent to the cell can affect coregulator function and transcriptional potency. As discussed above, coregulator concentrations are subject to turnover by ubiquitin-dependent and ubiquitin-independent proteasomal degradation pathways, whose activities can be abrogated by certain counteracting kinases. Therefore the cell concentrations and activation of degradation pathways for coregulators can play a role in SRM actions. In addition, in vivo systemic metabolism and selective cellular uptake or metabolism of ligands can sometimes modify SRM activities.

## WHAT ARE THE MOST IMPORTANT FACTORS FOR TISSUE-SPECIFIC SRM ACTIVITY?

The cell levels of activated coregulators are the primary determinant of tissue-specific SRM activity.[13] Having described herein the complete interacting equations and complexities of coregulator function, it remains that (1) the cellular complement of coregulators and (2) the cell and signaling context are the primary determinants of coregulator function. Consequently, they are the primary determinants of SRM functions.

SRMs are generally mixed antagonist/agonist ligands for receptors. When a receptor is occupied by a mixed antagonist/agonist ligand, the conformation generated in the receptor is neither purely antagonistic nor purely agonistic for activity. Rather, the conformation is intermediate for both functions (Fig 3). A pure agonist induces a receptor conformation that has a strong affinity for coactivators. A pure antagonist induces a receptor conformation that has a strong affinity for corepressors. The mixed antagonist/agonist ligand induces an intermediate conformation that, in turn, is intermediate in its affinity for both coactivators and corepressors. In other words, this receptor conformation is programmed by the local concentrations of activated coactivators and corepressors. The mechanism will obey the laws of physical chemistry. If the cellular concentration of preferred
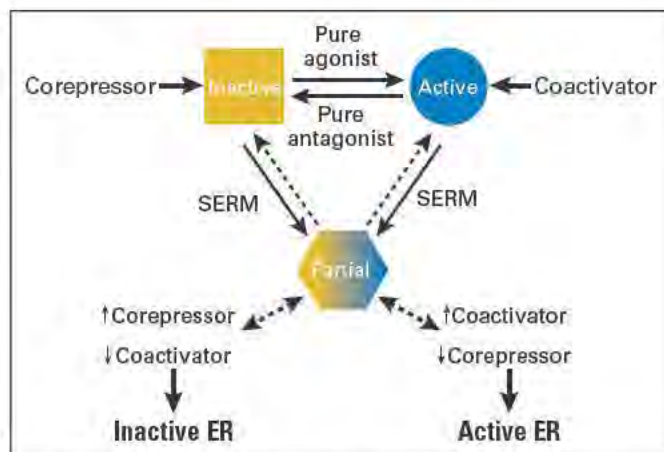


Fig 3. Hypothesis for tissue specific effects of selective estrogen-receptor modulators (SERMs). A schematic is shown for the contributions of coactivators and corepressors to the tissue-specific antagonist/agonist activities of a steroid receptor modulator (SRM). In the presence of a pure antagonist, a receptor is stabilized in the inactive conformational state and binds corepressor tightly. In the presence of a pure agonist, it conforms to a fully active conformation and binds coactivator tightly. In the presence of a mixed antagonist/agonist SRM, the receptor adopts an intermediate partial conformation that is neither fully inactive or fully active. In this intermediate conformation, the SRM-bound receptor is then even more subject to interactions with the relative intracellular concentrations of preferred coactivators or corepressors for its activity.

coactivators is high (or corepressors low), then the receptor is forced into the active conformation by the excess of coactivators and receptor dependent gene expression takes place. If the cellular concentration of preferred corepressors is high (or coactivators low), then the receptor is forced into the inactive conformation by the excess of corepressors and receptor-dependent gene expression is shut down. Since activation of coregulators occurs by post-translational modifications, the status of the cell signaling pathways that produces these post-translational modifications is an overlying modulator of SRM activity.

With this background of the physiologic basis for SERM action, it is now appropriate to meld these emerging data with the current applications of SERMs in the clinic and the evolving ideas about drug resistance to SERMs.

## CURRENT THERAPY WITH SERMs

The clinical application[5] of the laboratory strategy of long-term antihormonal therapy[37-39] as an adjuvant to treat breast cancer has now become the standard of care. Two approaches to antihormonal therapy have occurred during the last three decades: long-term treatment to block estrogen-stimulated growth at the level of the tumor ER[39] and, subsequently, the use of aromatase inhibitors to block estrogen biosynthesis in postmenopausal patients.[6] It is clear that the aromatase inhibitors offer advantages over tamoxifen as adjuvant treatments for postmenopausal patients; there are fewer adverse effects (blood clots and endometrial cancer), and aromatase inhibitors have a small but significant improved efficacy.[40,41] However, substantial numbers of postmenopausal patients continue to receive tamoxifen treatment either for economic reasons or because they are hysterectomized and at low risk for blood clots (low body mass index or they are athletically active). Postmenopausal women who have completed 2 to 5 years of adjuvant tamoxifen are also eligible for a further 5 years of antihormonal therapy with an aromatase inhibitor.[42-44] However, the veteran SERM tamoxifen is still the antihormonal treatment of choice for premenopausal patients and the antihormonal treatment for ductal carcinoma in situ (DCIS),[45] and remains the appropriate treatment to reduce breast cancer risk in premenopausal women at elevated risk.[46] It is important to stress that premenopausal women treated with tamoxifen do not experience elevations in endometrial cancer and blood clots, so the risk:benefit ratio is strongly in favor of tamoxifen treatment.[47]

The development of raloxifene[48] has created a new therapeutic dimension. Raloxifene is used either as a treatment and preventive for osteoporosis but with a quantifiable decrease in the incidence of breast cancer,[49,50] or as an agent for the reduction of breast cancer incidence in high-risk postmenopausal women.[51] The advantage of raloxifene as a SERM is that there are no increases in endometrial cancer[51,52] incidence previously noted with tamoxifen in postmenopausal women.[46,53]

The target site-specific actions of tamoxifen and raloxifene in breast and endometrial cancer were first noted in the laboratory,[54,55] but the question to be asked is why. On the basis of our earlier arguments about the mechanism of actions of SERMs, studies of the cellular context and coactivator content demonstrate the tissue-specific actions of tamoxifen and raloxifene in the uterine cancer cell.[56]

Overall, the SERM concept[10,11] clearly works in clinical practice, but the use of long-term SERM treatment regimens raises the important issue of the eventual development of drug resistance.

Laboratory studies have already shown that long-term SERM treatment changes the pharmacology from an antiestrogen- to SERM-stimulated growth.[57,58] This acquired resistance is a topic of immediate clinical concern.

## THE DIMENSION OF DRUG RESISTANCE TO SERMs

There are currently three possible mechanisms for drug resistance to tamoxifen. Either the patient can influence the effectiveness of tamoxifen via alterations in metabolism, or the ER-positive tumor is or can become refractory to treatment. These mechanisms are illustrated in Figure 4.

### Metabolic Resistance

The metabolic activation of tamoxifen occurs via demethylation to *N*-desmethyltamoxifen and subsequently transformation to the hydroxy metabolite endoxifen.[59,60] This topic has recently been reviewed[61] and will therefore be mentioned only briefly. Metabolic activation appears to be important for tamoxifen to acquire potent antiestrogenic and antitumor activity. Although large-scale prospective clinical trials have not been completed to prove the hypothesis definitively in large populations, there is sufficient preliminary data to warrant further study. Extensive laboratory studies demonstrate[62] that endoxifen is formed by the CYP2D6 enzyme system. However, there are wide variations in the CYP2D6 enzyme in the population that can influence drug metabolism. The wild-type CYP2D6 enzyme is referred to as CYP2D61*, whereas CYP2D64*/4* is a null variant. It is estimated that approximately 10% of the population have CYP2D6 variants, so the case can be made that these patients should be considered for other antiestrogenic interventions (eg, aromatase inhibitors). Another dimension for consideration is the control of menopausal symptoms, especially hot flushes. If tamoxifen is a prodrug and needs to be converted to endoxifen to achieve maximal antitumor activity at the tumor ER, then these same patients may have severe hot flushes. The selective serotonin reuptake inhibitors (SSRIs) have been found to be of value to treat hot flushes. The widespread use of tamoxifen as a long-term adjuvant therapy, especially in premenopausal patients, has naturally increased SSRI use. Unfortunately, the SSRIs such as fluoxetine and paroxetine are potent inhibitors of the CYP2D6 enzyme.[63] Therefore, symptom treatment has the potential to undermine the efficacy of tamoxifen if the incorrect SSRI is employed. Venlafaxine has a very low affinity for the CYP2D6 enzyme system and may be the agent of choice for treatment of hot flushes.[63] It should, however, be pointed out that there is no substantial clinical evidence to support this conclusion. A larger body of prospective clinical data is required to confirm the admittedly compelling preliminary studies.

### Intrinsic Resistance

A proportion of ER-positive tumors are intrinsically resistance to tamoxifen therapy. Historically, metastatic breast cancer that is ER and PR positive is approximately 80% responsive to antihormonal therapy (endocrine ablation or tamoxifen) whereas tumors that are ER positive but PR negative are only 40% responsive to antihormonal therapy.[64,65] We have known for about 20 years that enhanced growth factor signaling via the human epidermal growth factor receptor 1 (HER-1; EGFR) pathway impairs estrogen induction PR in breast cancer cells[66] and enhanced paracrine growth factor stimulation undermines that effectiveness of antiestrogen treatment at the ER.[67,68]
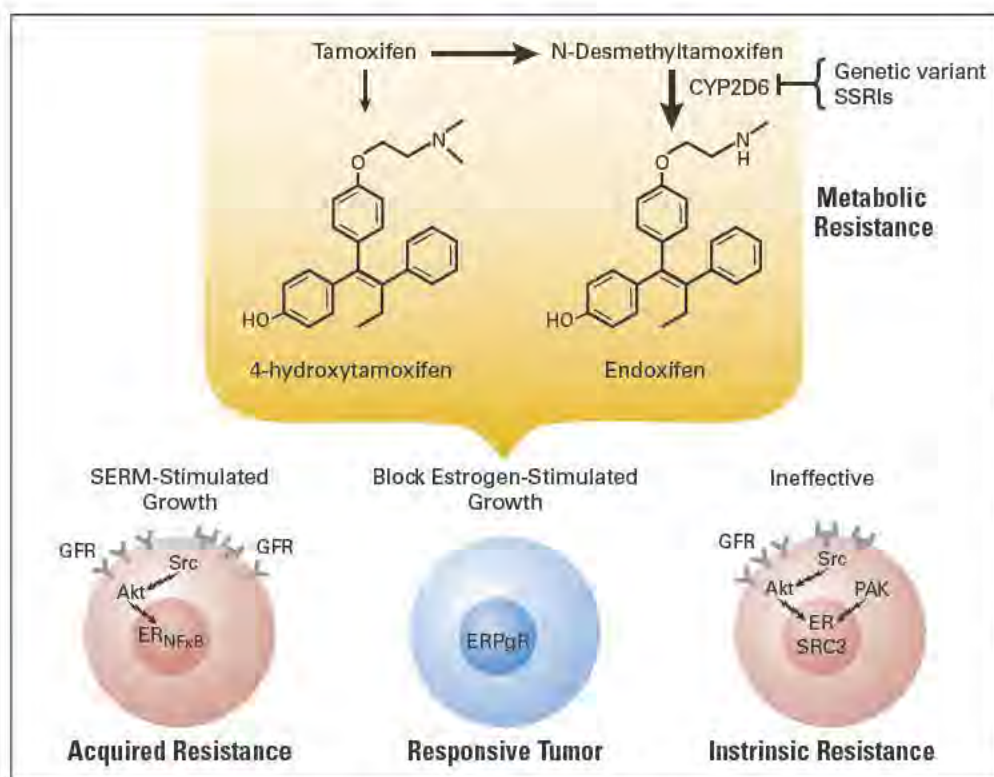
Fig 4. The possible types of drug resistance to the selective estrogen-receptor modulators (SERMs), particularly tamoxifen. Tamoxifen is a prodrug that needs to be metabolically activated by CYP2D6 to the active antiestrogen endoxifen. Mutation of CYP2D6 or the administration of specific selective serotonin reuptake inhibitors (SSRIs; eg, paroxetine or fluoxetine) to reduce hot flushes impairs metabolic activation and reduces the efficacy to tamoxifen. Tamoxifen and its hydroxylated metabolites are most effective at blocking estrogen-stimulated tumor growth if the cells contain both estrogen receptor (ER) and progesterone receptor (PgR). In contrast, tamoxifen is much less effective in controlling the growth of tumors, have high levels of membrane growth factor receptors (GFRs) that can activate phosphorylation cascades via Src, Akt and PAK. The ER and coactivator SRC3 could be targets for phosphorylation in these PgR-negative tumors. The tumor has intrinsic resistance to tamoxifen treatment. In contrast, tumors that initially respond to tamoxifen can acquire resistance to tamoxifen by increasing the level of GFR that phosphorylates Src and Akt. These SERM (tamoxifen)-stimulated tumors have increased nuclear levels of nuclear factor κ B (NFκB) but the tumors still rely on the ER for survival as second line treatments with either aromatase inhibitors (to block local estrogen production) or fulvestrant (to block the ER and cause preventative destruction) can result in the control of tumor growth.

These earlier observations have recently been confirmed and extended using breast cancer cells artificially transfected with insulin-like growth factor receptor[69] and using large tumor databases.[70] Tumor cell drug resistance to tamoxifen develops very quickly (8 weeks) in athymic mice with HER-2/*neu* engineered MCF-7 cells[71] compared with the natural process of more than 6 months.[57] Tamoxifen acts as an agonist in experimentally engineered breast cancer cells with high levels of the HER-2/*neu* growth factor receptor and the coactivator SRC3 (AIB1).[72]

In another approach, the possible connection between HER-2/*neu*, ER, PR and tamoxifen resistance has been evaluated in a tissue database linked to clinical outcomes. Intrinsic tamoxifen resistance is associated with HER-2/*neu*–, ER-positive, PR-negative tumors that have an increase in coactivator SRC3 (AIB1) levels.[73] Although the actual number is a small group of approximately 10% to 15% breast cancer patients, it does perhaps provide a clue to test who should avoid tamoxifen treatment.

The idea that growth factor receptor could be a predictor of SERM resistance has recently[74,75] been extrapolated to explain the reason for aromatase inhibitors being superior to tamoxifen as adjuvant therapy. A retrospective analysis[76] shows that patients with ER-positive, PR-negative tumors are more likely to respond to aromatase inhibitors than to tamoxifen. However, the conclusions, though attractive, require confirmation with prospective studies because of inconsistencies with the results from other direct trial databases comparing tamoxifen with an aromatase inhibitor and the recent reevaluation of the steroid receptor database in the original study of tamoxifen and anastrozole.[77]

### Acquired Resistance

Laboratory studies show that the treatment of athymic mice implanted with ER-positive, PR-positive MCF-7 tumors with continuous tamoxifen will eventually develop tamoxifen-stimulated tumors that will grow in response to either tamoxifen or estradiol.[57] Either no treatment or treatment with the pure antiestrogen fulvestrant[57,78,79] results in no tumor growth. Because no treatment in the ovariectomized athymic mouse is equivalent to treatment with an aromatase inhibitor and fulvestrant destroys the ER,[80] one could conclude that tumor growth is prevented in the absence of a stimulatory signal transduction pathway. This hypothesis is consistent with the clinical observation that anastrozole and fulvestrant treatment are equivalent after the failure of tamoxifen therapy.[81,82]

Goss et al[42] demonstrated that patients with ER-positive tumors and treated for 5 years with tamoxifen continue to be responsive to subsequent treatment with 5 years of the aromatase inhibitor letrozole.[83] This result could be interpreted as the slow development of acquired resistance by the breast cancer micrometastases during 5 years of tamoxifen so that these patients respond to a non–cross-resistant therapy that prevents tumor growth by blocking the ability of the patient to synthesize estrogen. Thus, the use of letrozole after tamoxifen is incrementally building on the already established long-term antitumor effect of tamoxifen that lasts for at least 10 years after the cessation of adjuvant therapy.[5]

## CONSEQUENCES OF LONG-TERM ANTIHORMONE THERAPY

Laboratory models of drug resistance should replicate the duration of SERM administration to patients. Most laboratory models of antihormone resistance are either engineered with stable transfection of the HER-2/*neu* gene into MCF-7 cells[72,84] or reflect the early development of resistance (SERM-stimulated growth)[57] to treatment. This later form of resistance is consistent with tamoxifen failure during the

treatment of metastatic disease. Under these clinical circumstances, tamoxifen treatment is effective for approximately 1 year. This form of SERM resistance is referred to as phase I.[85] However, tamoxifen is used as an adjuvant therapy for 5 years,[86] and it is reasonable to suggest that raloxifene will need to be administered for 10 years or more to maintain effectiveness as an antiosteoporosis medicine. Current studies[49] show that up to 8 years of raloxifene reduces the majority of (65%) but not all ER-positive breast cancers. Some tumors must, therefore, become raloxifene resistant.

The repeated transplantation of MCF-7 breast tumors into successive generations of tamoxifen-treated ovariectomized athymic mice for more than 5 years replicates the exposure of tumor cells to adjuvant tamoxifen. This approach to study SERM resistance results in a continuing dependence on tamoxifen to produce growth, but cross-resistance with the SERMs toremifene and raloxifene develops[79,87,88] and a significant change in the response of tamoxifen or raloxifene resistant cells to physiologic estradiol.[87,89,90] The signaling pathways for estrogen no longer support growth, but initiate apoptosis by inducing fas receptor, rapidly reducing levels of HER-2/*neu* and reducing nuclear factor $\kappa$ B (NF$\kappa$B) levels.[91] This form of SERM resistance is referred to as phase II resistance.[85] As might be expected, the pure antiestrogen fulvestrant can completely prevent tumor growth in animals. Paradoxically, when combined with physiological estrogen, fulvestrant not only reverses the apoptotic actions of estrogen but also causes robust tumor growth.[91] The mechanism for this therapeutically relevant observation is unclear, but may involve a dramatic upregulation of HER-2 and HER-3[92] but may also involve the recently described ligand (estrogen, SERM, fulvestrant) activator G protein GPR30.[93] It is possible that this novel observation may have value to plan an appropriate strategy to use fulvestrant plus an aromatase inhibitor as a third-line endocrine therapy.[94] The widespread clinical use of aromatase inhibitors now brings up the question of the consequences of the long-term use of aromatase inhibitors as adjuvant therapies. There will be an eventual development of drug resistance.

Early studies of estrogen deprivation in cell culture demonstrated that cellular ER levels and spontaneous cell replication increase.[95,96] Subsequent studies demonstrated that the cells initially become supersensitive to the growth properties of minute quantities of estrogen,[97,98] but as the duration of estrogen deprivation is extended, the cells respond to estrogen with the initiation of apoptosis.[99] This observation[99] has been used to explain the earlier application of high-dose estrogen therapy to treat postmenopausal women with metastatic breast cancer.[100] However, estrogen-deprived cell lines only need very low concentrations of estrogen in the postmenopausal range (lnM) to initiate apoptosis.[101,102] Cell death occurs through an increase in proapoptotic genes[103] and can be enhanced by specifically reducing the synthesis of bcl-2.[104] These preclinical studies are being translated to clinical trials by destroying phase II antihormone-resistant breast cancer cells with limited low-dose estrogen therapy followed by maintenance with further treatment with an aromatase inhibitor treatment.[103]

An alternate approach to study the development of drug resistance to aromatase inhibitors in vivo utilizes ER-positive MCF-7 breast cancer cells stably transfected with the CYP19 aromatase enzyme gene.[105] The cells grow into tumors in athymic mice treated with the enzyme substrate androstenedione that is converted to estrone.[106] The model has been used effectively to examine the integration of

SERM and aromatase inhibitor therapy and has effectively replicated the clinical experience.[107-110] Results not only clearly demonstrate the efficacy of aromatase inhibitors when compared with tamoxifen but also demonstrate the development of resistance to aromatase inhibitors.[111] Aromatase resistant tumors become more dependent on growth factor receptor pathways via mitogen-activated protein kinase.[112,113]

Overall, the basic knowledge of SERM action and the development of laboratory models of antihormonal resistance are proving invaluable to identify molecular targets for future advances in cancer therapeutics. Important clues about the pivotal role of SRCs in SERM drug resistance and tumor cell survival are already apparent. We predict that further progress in cancer cell biology will occur through an enhanced investment to understand the modulatory mechanisms of NRs and their coactivator partners. The new knowledge will create unanticipated opportunities to control cancer in the future.

## FUTURE POTENTIAL FOR NEW SRM DEVELOPMENT

With the advent of this recent knowledge of the molecular mechanisms of action of transcriptional regulators such as NRs and coregulators, new insights to drug development are rapidly becoming available. The discovery of tamoxifen as a SERM and the successful development of additional SERMs such as raloxifene, have encouraged exploitation of the SERM concept[10,11] by pharmaceutical companies to discover additional new SRM ligands for other NRs. Some examples are selective progestin modulators (SPRMs)[114,115] that inhibit uterine cancer but are devoid of stimulatory action in the breast; selective androgen receptor modulators (SARMs)[116,117] that are anabolic for muscle and bone, but spare the prostate; selective glucocorticoid receptor modulators (SGRMs)[118] that are strongly anti-inflammatory but do not induce glucose intolerance and connective tissue destruction; and selective peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) receptor modulators (SPARMs)[119-121] that promote insulin sensitivity.[13] All of the foregoing examples are under current development or are being tested in clinical trials. In the case of each of these SRMs, the molecular mechanisms and pathways for their efficacy described herein represent the guiding principles for their tissue-specific actions and represent a substantial health care return for the investment in basic mechanistic scientific research.

## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

## AUTHOR CONTRIBUTIONS

**Conception and design:** V. Craig Jordan, Bert W. O'Malley
**Administrative support:** V. Craig Jordan
**Provision of study materials or patients:** Bert W. O'Malley
**Collection and assembly of data:** V. Craig Jordan, Bert W. O'Malley
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# Special Review

# Optimizing the Antihormonal Treatment and Prevention of Breast Cancer

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The incidence of breast cancer is rising throughout the world. Breast cancer is slowly becoming more prevalent in countries which previously had low rates of cancer as well as becoming a leading cause of cancer death in some countries. Fortunately, a large number of these tumors are estrogen receptor (ER) positive and respond to anti-hormonal adjuvant therapy which until recently has been 5 years of tamoxifen treatment. Unfortunately, a significant number of patients develop recurrent cancers and the recurrent tumors are resistant to tamoxifen treatment. In addition, because of tamoxifen's selective estrogenic actions, there have been reports of venous thrombosis, endometrial cancer, and strokes in patients receiving tamoxifen therapy. Thus, there are other novel therapies such as aromatase inhibitors that block estrogen production in postmenopausal women or fulvestrant that destroys the estrogen receptor. This paper will summarize the therapeutic options for anti-hormonal therapy, the role of anti-hormonal agents in advanced breast cancer, and adjuvant therapy and the current status of chemoprevention with selective ER modulators.

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Key words: Tamoxifen, Aromatase inhibitors, Fulvestrant, Estrogen receptor

## Introduction

There are approximately 1,000,000 new cases of breast cancer in the world each year[1]. Unfortunately, as we increase our understanding of the biologic behavior of these tumors, the incidence of breast cancer continues to rise throughout the world.

There are currently over 2 million breast cancer survivors in the United States. This year, there will be an estimated 212,920 new cases of invasive breast cancer with the rate of invasive breast cancer increasing by 0.3% per year since 1987[2]. According to the surveillance, epidemiology, and end results (SEER) from the 1998-2002 National Institutes of Health databases, there was an incidence of 501.8 cases of breast cancer per 100,000 women in the United States during this time period. The mortality rate was 103.8 per 100,000 women[2]. On a stage by stage basis, 55%

of women had stage 0-1 breast cancer. The next largest group had stage 2 breast cancer and constituted 30% of the patients. Stage 3, 4, and unstaged breast cancer each constituted 5 % of breast cancer patients[3].

In Japan, the incidence of breast cancer is much lower than the United States. However, the incidence has been rising steadily. Since 1975, the incidence of breast cancer has more than doubled. In 1975, approximately 15/100,000 women developed breast cancer. By the year 2000, approximately 45/100,000 women developed breast cancer. The majority of the women with breast cancer were 50 years of age or older[4]. Additionally, from 1970-1999, out of approximately 15 million women who were screened, 2,340 cases of breast cancer were detected. In the time period between 1999-2000 alone, there were 1,168 new cases of breast cancer out of 986,913 patients screened[5]. In 2001, approximately 9,654 women died of breast cancer in Japan[6]. By the year 2020, the Japanese Cancer Registry estimates that the annual incidence of breast cancer will be 127,000 in Japan if current trends continue[7].

The discrepancies in breast cancer incidence and the trends in an increased incidence in Japan

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lead one to question the underlying cause. Throughout history, scientists have noted that breast cancer occurred at a higher frequency in nuns and nulliparous women. Also, first childbirth at a later age correlates with breast cancer risk. In western countries, childbearing occurs at a later age. Traditionally, Japanese women tend to have more children and start having children at a younger age. Obesity and diets high in saturated fats such as American diets also correlate with higher rates of breast cancer. Traditional Japanese diets are 10-25% fat, whereas U.S diets are 40-45% fat[8]. In the past decade, more Japanese women are having children at a later age, fewer children, and have adopted more of a "western" diet which may explain the rise in breast cancer incidence[9]. What does this mean? Progesterone causes maturation of the glandular breast tissue during pregnancy, so early pregnancy can be viewed as chemoprevention. In nulliparous women, the breast tissue is exposed to unopposed estrogen, which causes proliferation of the gland. Higher fat stores in the body lead to higher levels of peripheral estrogen, particularly during the postmenopausal period. Thus, estrogen can be viewed as promoting estrogen responsive breast cancer development. In the United States, this is evidenced by a rise in estrogen receptor (ER) positive breast cancer and a decrease in ER negative breast cancers[10]. Since 1990, the SEER database in the United States has been updated to include ER positive and ER negative breast cancers. Out of 82,488 breast cancer patients from the 1992-1998 databases, 25% of the women had ER negative cancer and 75% of the women had ER positive cancer. Of those patients with ER negative cancer, 21% were greater than 50 years of age, while 37% were less than 50 years of age[11].

In Japan, a few small studies have been undertaken at various hospitals looking at cases from the 1970's to determine the percentage of ER positive cancer. No recent studies have been found. One early study demonstrated that 55% of 456 patients had ER positive breast cancer. The distribution was similar between pre-menopausal and post-menopausal patients[12]. Another paper compared international studies of ER positive cancer in the 1970's[13]. The rate of ER positive breast cancer in a study from 1977 was 58% (1060 patients) in Japanese and 71% in American women. The patients were further categorized into premenopausal and postmenopausal patients. At this time,

57% of premenopausal Japanese women had ER positive cancer and 59% of premenopausal American women studied had ER positive cancer. The discrepancy in ER positive status was primarily between postmenopausal patients in both countries. Postmenopausal Japanese women had 59% ER positivity, while 71% of postmenopausal American women were ER positive[14]. Another study compared 260 patients from Japan with 410 patients in Western countries. The regression rate of ER positive tumors to endocrine therapy was 48% and 55%, respectively[15]. Regardless of the difference in ER positive tumors, the response to endocrine therapy was similar.

Clearly, it would be interesting to compare the current incidence of ER positive tumors between Japan and Western countries. Since the incidence of breast cancer is rising in Japan and many Japanese women have adopted "western" lifestyles, one would hypothesize that the incidence of ER positive breast cancer has risen in Japan.

The pioneering work of Elwood Jensen[16] identified the ER as the signal transduction pathway that controls the growth of the majority of breast cancers. The ER subsequently became the therapeutic target for the development of antiestrogenic drugs[17]. Thus, antihormonal therapy plays an important role in the therapeutic armamentarium for high risk patients and those patients with a diagnosis of breast cancer. Several established options to treat ER positive breast cancer are now available since the introduction of tamoxifen to Japan more than 20 years ago.

### The Strategical Application of Endocrine Therapy:

Estrogen plays a key role in the development and proliferation of milk glands in the breast. Until recently, it was generally accepted that estrogen does not directly cause breast cancer. However, recent laboratory studies indicate that estrogen has an oncogenic action in breast cells[18, 19]. Nevertheless, because of the strong signal that estrogen has on mediators of the cell cycle, cells that have oncogenic mutations may continue to divide in the presence of estrogen[20]. Eventually, these cells develop into a palpable tumor. For patients who have ER positive breast cancer, there are important therapeutic options a clinician can implement based on the well developed strategy of anti-estrogenic therapy.

The intent of antihormonal therapy in women
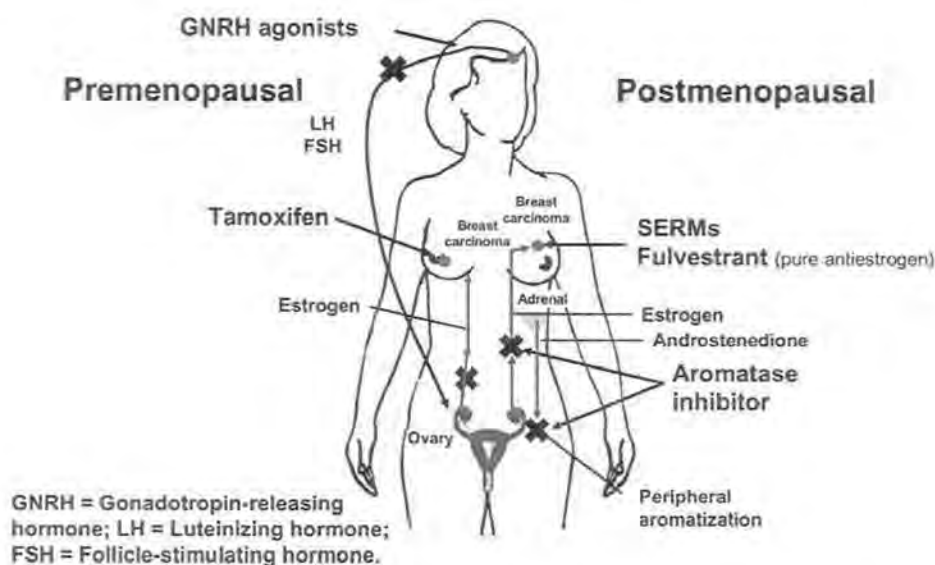
Fig 1. The sites of action of various classes of endocrine agents used to treat ER positive breast cancer. SERMs such as tamoxifen and toremifene inhibit breast cancer cell proliferation and act as antagonists in the breast. Aromatase inhibitors block the aromatization of androstenedione into estradiol in peripheral tissues, hence preventing production of estrogen. GNRH agonists such as goserelin prevent release of luteinizing hormone and thus inhibit the activation of estrogen production in the ovary. Fulvestrant destroys the ER in breast cancer cells and prevents ER mediated cell replication.

is based upon blocking the ER signal transduction pathway or inhibiting the synthesis of estrogen (Fig 1). One option is to use selective estrogen receptor modulators (SERMs) that block the ER and stop breast cancer growth. Tamoxifen, the prototypical SERM, exerts its effect by binding to the ER receptor and altering the conformation of the complex, thereby inhibiting signal transduction cascades that stimulate cell replication. Unfortunately, tamoxifen expresses its SERM action by promoting cell replication in endometrial cancer cells. This is an estrogen like action that results in a small but significant increase in the detection of endometrial cancer during long term adjuvant therapy or when used in chemoprevention. Nevertheless, it is important to stress that the increase in endometrial cancer noted with tamoxifen only occurs in postmenopausal women[21, 22]. Tamoxifen remains the treatment of choice for premenopausal patients.

An alternative to SERMs is to block the synthesis of estrogen in the body with an aromatase inhibitor. Anastrazole, letrozole, and exemestane have all been shown to be effective in treating advanced breast cancer in postmenopausal women. These drugs inhibit the synthesis of estrogen in peripheral tissues[23]. It is important to stress that aromatase inhibitors should not be

used in premenopausal women because ovarian estrogen production may resume through feedback mechanisms[24]. Finally, another option for hormonal therapy in post-menopausal women with advanced breast cancer and recurrent cancer is to use an ER down-regulator called fulvestrant. Fulvestrant is administered as a monthly injection to provide a slow, continuous release of the drug to the patient. The active drug binds to and alters the shape of the ER in the tumor cell. The peculiar shape of the fulvestrant-ER complex then results in the rapid destruction of the ER. As a result of the destruction of the ER signal transduction pathway, fulvestrant has no agonist activity on the ER[25].

An antihormonal option for premenopausal women is the long acting gonadotropin releasing hormone, goserelin. The sustained release of goserelin from the implantation of a rice grain sized depot lasts for one month and desensitizes the hypothalamic-pituitary axis to produce the equivalent of a medical oophorectomy[26].

In this paper, we will discuss the agents currently available in the United States to treat ER positive breast cancer. We will first discuss the use of antihormonal agents to treat metastatic breast cancer. This will serve as a basis for our discussion of current adjuvant therapies and

recent results of chemoprevention studies.

### Tamoxifen, the Pioneer for Hormonal Therapy for Advanced Breast Cancer and Adjuvant Therapy

The treatment for advanced breast cancer has changed dramatically over the past three decades. Initially, advanced breast cancer therapeutics focused on nonspecific cytotoxic agents. The reinvention of tamoxifen from a failed "morning after pill" to the first targeted therapy for breast cancer provided the clinical research community with an invaluable new therapeutic tool to pioneer the strategy of long term antihormonal therapy and chemoprevention[27]. Not surprisingly, enthusiasm from the medical community and pharmaceutical industry was not high. The early trials have been summarized with approximately a 30% response rate in affected patients[28]. This improves if ER positive patients are selected for targeted treatment. Tamoxifen responses were the same as any other endocrine approach to breast cancer; however, the advantage of tamoxifen was the low incidence of side effects compared to other endocrine therapies[29].

The use of tamoxifen as the pioneering agent as an adjuvant to surgery has its origins in studies from the 1970's[27]. The laboratory studies demonstrated the feasibility of targeting the ER and using long term anti-hormonal therapy[30, 31] so that tamoxifen could be reasonably considered for use as an adjunct to surgery in node positive and then node negative patients. The reason for using tamoxifen as an adjuvant following surgery is to prevent recurrence. The clinical trials ultimately showed both increases in disease-free and overall survival with adjuvant tamoxifen treatment[20]. The studies demonstrated that there is a 50% decrease in recurrence in ER positive patients with 5 years of tamoxifen. Even 15 years after a diagnosis of ER positive breast cancer, treatment with 5 years of tamoxifen continues to decrease mortality[32]. However, there is no increase in disease free or overall survival with ER negative cancer.

The transition of tamoxifen from a short term treatment to a long term therapy for node positive and node negative breast cancer increased awareness of the pharmacology and side effects of tamoxifen[33]. Tamoxifen is a SERM, so the drug preserves bone density and potentially reduces the risk of fractures secondary to its agonist effects on the ER receptors in bone. Additionally, tamoxifen decreases circulating cholesterol, but this side effect is thought to greatly improve patient prognosis. The negative side effects include an increase in the incidence of endometrial cancer and venous thrombosis. However, the benefits of tamoxifen treatment greatly outweigh the risks of endometrial cancer and venous thrombosis[32].

The ubiquitous use of tamoxifen in the treatment plan for breast cancer has improved patient prognosis and enhanced survivorship dramatically. Nevertheless, the knowledge of the estrogen-like side effects of tamoxifen has focused research efforts on understanding tamoxifen induced drug resistance and the development of new and safer agents to treat breast cancer.

### Drug resistance to Tamoxifen:

Resistance to therapy is most often observed during the treatment of advanced breast cancer. After 1-3 years of tamoxifen treatment, breast tumors start to grow despite continuing tamoxifen therapy. However, what is unique about tamoxifen resistant tumors is that a withdrawal response occurs if tamoxifen treatment is stopped[34]. The tumor is dependent on tamoxifen. This phenomenon is best illustrated in the laboratory. Animal models have demonstrated that ER + breast cancer lines can eventually develop resistance to tamoxifen and the tumors then grow in response to tamoxifen[35]. The resistance to tamoxifen is believed to occur because of an increase in cell surface signaling through the HER2/neu/EGFR or Insulin like growth factor receptors that promote phosphorylation of the ER and its coactivators[36]. This in turn activates breast cancer cell growth (Fig 2). Based on the recognition that tamoxifen has limitations and that less estrogenic like drugs would be useful therapeutic agents, it is reasonable to examine the rational application of aromatase inhibitors and fulvestrant.

### Second Line Therapy Following the Failure of Tamoxifen

In addition to stopping tamoxifen, there are multiple ways to address failure of tamoxifen therapy and subsequent resistance to tamoxifen (Fig 3). At present, the first two options are hypothetical. Clinicians have an intense interest in the feasibility of blocking the cell surface receptors with monoclonal antibodies or blocking the tyrosine kinases that cause tamoxifen resistance. At present there are suggestions that blocking the HER2/
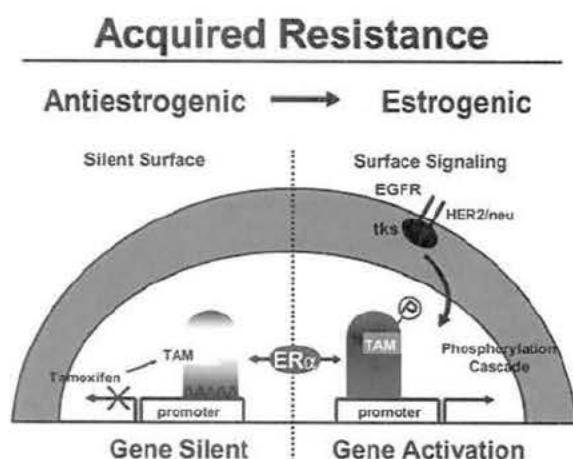
**Fig 2.** The proposed mechanism of tamoxifen resistance. With a silent surface, tamoxifen successfully prevents ER stimulated proliferation by altering the shape of the ER. With surface signaling, an increase in cell surface signaling through the HER2/neu/EGFR receptor promotes phosphorylation of the ER. This leads to proliferation of breast cancer cells in the presence of tamoxifen or estrogen.

neu pathway has promise, but only in patients with gene amplification. In contrast, the second two options for second line therapy are based on the mechanism of tamoxifen resistance. Laboratory studies show that tamoxifen resistant tumors will grow both with tamoxifen and estrogen[35]. Estrogen is produced by aromatization in postmenopausal women. Therefore, a plan to stop the reactivation of tumor growth after tamoxifen is reasonable. Aromatase inhibitors are the agent of choice to create a "no estrogen" state[37, 38]. Fulvestrant can also be used to destroy the estrogen receptor completely. In the absence of the ER, estrogen stimulated proliferation cannot occur, regardless of the cellular signaling mechanisms that activate the ER. Clinical studies of second line therapies after the development of tamoxifen resistance show that anastrazole and fulvestrant are equally effective in controlling breast tumor growth[39, 40]. Thus, for the purposes of clinical clarity, the treatment paradigm for patients who fail SERM therapy can be summarized as shown in figure 4.

### Adjuvant Therapy with Aromatase Inhibitors

Since aromatase inhibitors do not have the estrogen-like side effects noted with tamoxifen and there is clinical evidence that they can be used once resistance to tamoxifen occurs, the logical question arises of whether they are an improvement over tamoxifen in the clinical setting. Five different studies (Fig 5) have shown
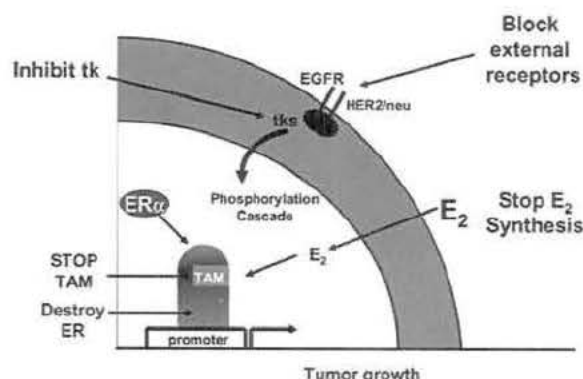


**Fig 3.** Possible strategies to prevent ER receptor activation in SERM resistant breast cancer. Herceptin, a monoclonal antibody blocks Her2/neu/EGFR to prevent phosphorylation of the ER. Alternatively, a number of small molecules that inhibit the receptor tyrosine kinase are being explored. Aromatase inhibitors prevent the formation of estrogen in a postmenopausal woman's body. Without estrogen available to bind the receptor, the tamoxifen resistant cells cannot proliferate. Another option is to destroy the receptor completely with fulvestrant, a pure antiestrogen that binds to the ER. The strange shape of the complex programs the ER complex for rapid destruction. Thus, there will not be any ER mediated replication of cancer cells.

that the aromatase inhibitors, anastrazole, letrozole, and exemestane are better than tamoxifen at preventing contralateral breast cancer, improving disease-free survival, decreasing the risk of endometrial cancer, and have no additional risk of blood clots. In all of the studies, the patient demographics, stages of cancer, and hormone receptor status were well matched between control and experimental groups. The primary endpoint was locoregional and distant recurrences. Disease-free survival was compared between treatment and control groups as well as side effect profiles, adverse events, and deaths related or unrelated to breast cancer.

Several questions were addressed by the different studies. One question that was addressed by Boccardo *et al.*[41] was whether or not switching to anastrazole after two to three years of tamoxifen would help prevent relapse. The median follow up time was 36 months. One group received tamoxifen for 5 years (225 patients) and the other group received tamoxifen for 2-3 years followed by anastrazole for 3 years (223 patients). Patients who switched to anastrazole had a longer disease free survival. The difference between switching to anastrazole and continuing tamoxifen was 5.8%[41]. In addition, another larger international study examined the use of exemestane after 2-3 years of
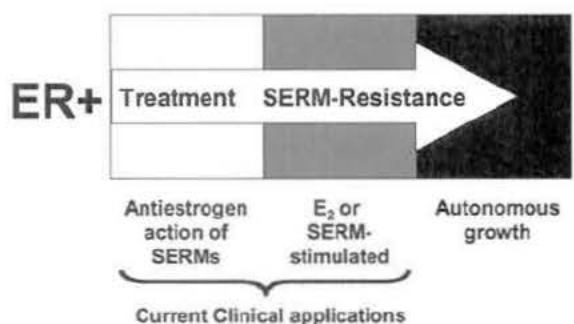
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Fig 4. The development of SERM resistance noted in the clinical setting. During the treatment phase, tamoxifen effectively blocks ER mediated cell proliferation. During the development of tamoxifen resistance, estrogen or SERMs can stimulate breast cancer cell growth. The cancer cells need tamoxifen or estrogen to grow. Finally, resistant breast cancer cells can acquire autonomous growth (ie. The tumor is not dependent on either estrogen or the SERM for survival.

## Long Term Estrogen Deprivation Treatment



Fig 5. A summary of all 5 of the major trials that were conducted to compare tamoxifen and aromatase inhibitors as adjuvant therapy following surgery. The first group examined the use of aromatase inhibitors instead of 5 years of tamoxifen. The next group compared 5 years of tamoxifen with 2-3 years of tamoxifen followed by 3 years of an aromatase inhibitor. The last group compared 5 years of tamoxifen followed by 5 years of aromatase inhibitors.

tamoxifen (2380 patients) vs. continuing tamoxifen (2362 patients). The design was similar to the study design by Boccardo *et al.* and the disease free survival was improved by 4.7 % when patients were switched to exemestane[42].

However, the question has been asked "can an aromatase inhibitor improve prognosis after the full five years of tamoxifen treatment?" Goss and his coworkers have compared letrozole (2575 patients) and placebo (2582 patients) after 5 years of tamoxifen in breast cancer survivors. The median follow up was 2.4 years and the disease free survival was 93% in the letrozole group and 87% in the placebo group, with an absolute difference of 6%[43].

Since there are benefits to switching to aromatase inhibitors, why not use an aromatase inhibitor immediately following surgery? This next question was addressed by two large multinational clinical trials. The ATAC trial compared anastrazole, tamoxifen or a combination of tamoxifen and anastrazole to see if disease free survival improved[44]. There were over 9000 patients enrolled to receive tamoxifen alone (3116 patients), anastrazole alone (3125 patients), or anastrazole plus tamoxifen (3125 patients). The disease free survival after 3 years of anastrazole, tamoxifen, or a combination of the two was 89.4%, 87.4%, and 87.2%. Additionally, after 3 years, hazard ratios favored anastrazole over tamoxifen for node negative and node positive disease (0.85 for node positive and 0.7 for node negative)[44]. Since there was no difference between the combination arm and

the tamoxifen arm, the combination arm was closed and the anastrazole (n=2618) and tamoxifen patients (n=2598) were followed further for 2.7 years. While overall survival was the same between the two groups, the disease free survival (absolute difference of 3%), time to recurrence (absolute difference of 3.7%), and time to distant recurrence (absolute difference of 2%), were better in the group that received anastrazole vs. tamoxifen. Patients who received anastrazole (n=3092) instead of tamoxifen (n=3094) had a decrease in the risk of contralateral breast cancer (35 vs. 59 patients). In addition, the patients who took anastrazole also had a decrease in endometrial cancer (5 vs. 17 patients) and blood clots (87 vs. 140 patients)[45].

The initial portion of the BIG study addressed the use of letrozole (n=4003) in comparison with tamoxifen (n=4007)[46]. An additional study will compare letrozole followed by tamoxifen, and tamoxifen followed by letrozole. At five years, patients taking letrozole had less recurrence than those on tamoxifen as demonstrated by an absolute difference of 3.4%. In the letrozole group, 16 patients developed a contralateral breast cancer, while 27 patients in the tamoxifen group developed a contralateral breast cancer. Most importantly, the BIG trial further stratified patients into node negative and node positive groups as far as disease free survival rates. When looking at dis-

ease-free survival alone, the patients with node positive cancer benefited the most from letrozole (hazard ratio= .71). The 5 year disease-free survival was 77.9% in those patients who received letrozole vs. 71.4% in those who received tamoxifen. Patients with node negative cancer had an 88.7% rate of disease-free survival (hazard ratio of 0.96) regardless of the use of letrozole or tamoxifen. Fewer women had endometrial cancer in the letrozole group (4/3089 patients) when compared to those patients taking tamoxifen (10/3157 patients). In addition, the patients taking letrozole had a significant decrease in thromboembolic events (61/3975 patients) in comparison to the patients who received tamoxifen (140/3988)[46].

Overall, the trials have shown that aromatase inhibitors are favorable to tamoxifen in postmenopausal, ER positive patients, secondary to decreased recurrence and a decrease in undesirable side effects such as endometrial cancer and venous thrombosis. The concern with aromatase inhibitors is the higher rate of osteoporosis and subsequent fractures as well as higher serum cholesterol levels. Practitioners must be proactive and monitor patients for joint pain and do regular cardiovascular risk stratification. Fortunately, there are medicines such as statins to lower cholesterol and bisphosphonates to maintain bone density that can now be a part of the patient's treatment plan.

### Is Prevention Better Than Cure?

The antihormonal therapy of breast cancer has probably reached its zenith, but the application of SERMs for the chemoprevention of breast cancer is providing a new dimension for the consideration of public health. Despite progress in the treatment of breast cancer, prevention remains a viable strategy. In the 1970's laboratory studies showed that tamoxifen prevented carcinogen induced rat mammary cancer, probably via an ER mediated mechanism[47]. Gradually, this work was translated to clinical trial and the results demonstrated that tamoxifen would be a useful agent to test as a chemo-preventative[23]. The NSABP-1 trial subsequently showed that in high risk women, tamoxifen significantly decreased the risk of breast cancer by 50% in pre and postmenopausal volunteers. Tamoxifen is approved in the United States for risk reduction of breast cancer in high risk premenopausal and postmenopausal women. Despite the fact that chemoprevention is a pioneering

application for tamoxifen, there are justifiable concerns about toxic side effects. The side effects (primarily venous thrombosis and endometrial cancer, though the incidence is small) have limited the use of tamoxifen by primary care practitioners. Nevertheless, it must be stressed that the side effects are limited to postmenopausal women. There are no significant increases in endometrial cancer and thrombosis in postmenopausal women. Tamoxifen remains the chemo-preventative agent of choice in this risk group[48, 49]. To address the issue of side effects (e.g. endometrial cancer) with tamoxifen, a novel SERM strategy was devised. If SERMs can prevent bone loss and prevent recurrent cancer at the same time[50, 51], why not develop a SERM to prevent osteoporosis and prevent breast cancer at the same time? This evidenced based hypothesis[52] has now been evaluated with the application of raloxifene to prevent osteoporosis. Breast cancer and endometrial cancer are reduced when raloxifene is used to treat osteoporosis[53, 54]. It is now possible to state that thousands of women treated with raloxifene to prevent osteoporosis will have significant reductions in their breast cancer incidence[49, 55, 56]. Based on the successful evaluation of raloxifene as an osteoporosis drug, raloxifene was then targeted to postmenopausal women at high risk for breast cancer. The studies of tamoxifen and raloxifene (STAR) trial was designed to compare and contrast tamoxifen and raloxifene for the reduction in the incidence of breast cancer in high risk women and to improve the side effect profiles[57]. The results of the trial demonstrated that the SERMs are equivalent in the prevention of invasive breast cancer, but it appears that tamoxifen is slightly better in preventing noninvasive breast cancer. Nevertheless, the results do not reach statistical significance. The side effect profile of raloxifene is better than tamoxifen. Raloxifene treated women have a reduced incidence of endometrial cancer, hysterectomies, cataracts and cataract surgery. Overall, raloxifene can be stated to be an effective agent to reduce breast cancer risk in postmenopausal women.

It is important to emphasize that no other classes of hormonal therapy have been evaluated successfully for prevention other than SERMs. Aromatase inhibitors are slightly superior to SERMs for patients with breast cancer. Unfortunately, aromatase inhibitors cannot be used in premenopausal women. Therefore, the future consid-

eration for the aromatase inhibitors is the balance of side effects between SERMs and aromatase inhibitors. The side effect profile of osteoporosis, fractures, and musculoskeletal pain in high risk patients will be addressed by an international clinical trial by the International Breast Intervention Study (IBIS II). Anastrazole is being evaluated for prevention in high risk post menopausal women. The study involves 6,000 women who will be randomized to receive anastrazole or placebo. Another group of 4,000 women with locally excised DCIS will be randomized to receive anastrazole or tamoxifen. After 5 years of treatment, rates of breast cancer and side effect profiles will be examined between the groups[58]. The important trial (P4) to compare and contrast raloxifene with the aromatase inhibitor letrozole is being conducted by the National Surgical Adjuvant Breast and Bowel Project (NSABP). These data will not be available until 2014.

*Overall Summary*

When treating patients with ER positive breast cancer, there are many new choices available for patients. Recurrent cancer which is SERM resistant and side effects such as venous thrombosis and endometrial cancer prompted investigation into aromatase inhibitors. Currently, clinical trials indicate that aromatase inhibitors should be used for initial hormonal therapy. One must keep in mind that although aromatase inhibitors are superior for prevention of recurrence, some patients still get recurrent breast cancer and the absolute difference between aromatase inhibitors and tamoxifen is a small percentage when comparing individual studies. Furthermore, the BIG trial is the only trial that stratified patients into node negative and node positive patients in terms of disease free survival rates. Letrozole was significantly more beneficial with node positive patients, and there was no difference between node negative patients. If fractures, bone pain and other side effects are intolerable, tamoxifen is still a viable option for prevention of recurrence, especially in node negative patients with no uterus and without a history of clotting. In node positive patients, aromatase inhibitors are probably the best initial treatment option. Treatment for recurrent cancer should be tailored for individual patients based on disease characteristics and tolerance of side effects such as osteopenia and musculoskeletal pain, cardiac risk, risk of venous thrombosis, and

risk of uterine cancer. For the future, prevention of breast cancer remains the most ideal situation and a significant number of patients will continue to benefit from the use of tamoxifen or raloxifene, with an additional benefit of maintaining bone density. The era of multifunctional medicines has arrived.

## Acknowledgements

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# SERMs for the treatment and prevention of breast cancer

**Ramona F. Swaby · Catherine G. N. Sharma ·
V. Craig Jordan**

**Abstract** Tamoxifen and raloxifene are both selective estrogen receptor modulators (SERMs). The medicines can block estrogen mediated breast cancer growth and development but will also maintain bone density in postmenopausal women and lower circulating cholesterol. Tamoxifen has remained the antihormonal therapy of choice for the treatment of ER positive breast cancer for the last 30 years. However, although adjuvant tamoxifen produces profound increases in disease-free and overall survival in patients with ER positive breast cancer, concerns about drug resistance, blood clots and endometrial cancer have resulted in a change to the use of aromatase inhibitors for the treatment of postmenopausal women. Nevertheless, tamoxifen remains the antihormonal treatment of choice for premenopausal women with ER positive breast cancer and for risk reduction in premenopausal women who are at high risk for developing breast cancer. The risk of endometrial cancer and thromboembolic disorders during tamoxifen therapy is not elevated in premenopausal women. It is important to note that aromatase inhibitors or raloxifene should not be used in premenopausal women. Raloxifene is used to prevent osteoporosis in postmenopausal women and, unlike tamoxifen, does not increase the risk of endometrial cancer. However, raloxifene does reduce breast cancer risk by 50–70% in both low risk and high risk postmenopausal women. Comparisons of raloxifene with tamoxifen show equal efficacy as a chemopreventive for breast cancer but there is a reduction in thromboembolic disorders, fewer endometrial cancers, hysterectomies, cataracts and cataract surgeries in women taking raloxifene. Overall, SERMs continue to fulfill their promise as appropriate medicines that target specific populations for the treatment and prevention of breast cancer.

**Keywords** Tamoxifen · Raloxifene · Estrogen receptor · Selective estrogen receptor modulator · Osteoporosis · Endometrial cancer

## 1 Introduction

Schinzinger [1, 2] first proposed, whereas Beatson [3, 4] first reported, performing oophorectomy for the treatment of metastatic breast cancer in 1896. It has now become accepted that ovarian hormones, particularly estrogen, are central to the development of breast cancer. Laboratory evidence identified estrogen as the trophic hormone in estrogen target tissues (e.g. the uterus and some breast cancers) [5] so naturally "anti-estrogen" therapy became a central theme for the treatment and now prevention of breast cancer [6] One medicine, tamoxifen [7], originally classified as a nonsteroidal antiestrogen [8] but now reclassified as a selective estrogen receptor modulator (SERM) [9] has proved to be a pioneering intervention that not only produced dramatic survival advantages when used as an adjuvant therapy [10] but also became the first chemopreventive for any cancer [11, 12]. However, the recognition of SERM action [13, 14] actually opened the door to new opportunities in therapeutics and advanced the idea of multifunctional medicines to address a number of prevention issues pertinent to postmenopausal women's health. Osteoporosis is a major health care problem but emerging information about the inappropriateness of long-

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term hormone replacement therapy (HRT) to prevent osteoporosis has acted as a catalyst for the development of new, safer SERMs.

In the United States alone, approximately 90 million prescriptions for HRT were dispensed annually from 1999 through 2002 [15]. Indeed, records suggest that hormonal replacement therapy was the most commonly prescribed medicine in the world during the late 1990s and early 2000s [16]. Despite epidemiologic data suggesting the overwhelming benefits of HRT, data regarding hormonal therapy use and breast cancer incidence were unconfirmed. Therefore, as part of the Women's Health Initiative (WHI), a large randomized controlled primary prevention trial to determine the risk benefit ratio of HRT in postmenopausal women was undertaken. In July of 2002, the principal results from the WHI study examining the effects of HRT were reported [17]. In this trial in which approximately 16,000 women were treated either with estrogen/progesterone combination HRT or placebo, an approximately 26% increase in the incidence of breast cancer was detected among the women treated with HRT. This data was subsequently confirmed and extended in the Million Women Study [18]. The Million Women Study, while not a randomized prospective clinical trial, followed cohorts of post-menopausal women during the same time frame as the WHI and collected information about their use of HRT. These cohorts were followed for cancer incidence and any death due to breast cancer. The overall conclusion was that users of HRT were more likely than never users of HRT to develop breast cancer and die from it [18]. The profound excess of new breast cancers that accumulated populations of women taking 5 or 10 years of HRT is illustrated in Fig. 1. As soon as these data were reported, the use of HRT dropped dramatically both within the United States and in Europe [15, 19].

Recently, a 7% decrease in the age-adjusted incidence of breast cancer has been observed from 2002–2003 [20]. This
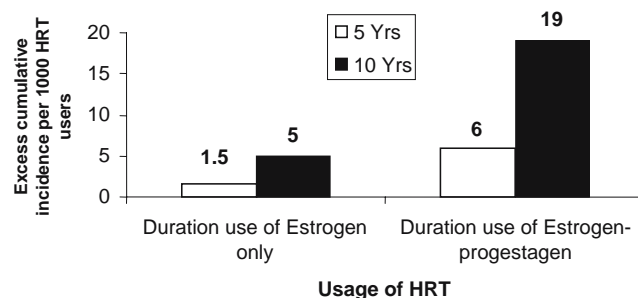


**Fig. 1** Diagramatic representation of the estimated cumulative incidence of breast cancer in 1,000 postmenopausal women taking hormone replacement therapy (HRT) in excess of the incidence observed in women not taking HRT. The Million Women's Study [18] compared and contrasted women using estrogen only preparations for 5 or 10 years with those women taking a combination of estrogen and a progestin for the same period

decline, not attributable to changes in mammography screening, represents a decline of approximately 14,000 breast cancer cases in the United States in 2003 when compared with 2002. The effect was found to be important for women age 50 or greater and specifically, statistically significant for women aged 50–74. Most importantly, this effect was essentially confined to hormonally responsive breast cancers. While these data do not speak to the initiation and development of breast cancers, the time course suggests that estrogen may play a role in propagating sub-clinical ER breast cancers that in a less estrogenic environment may have remained sub-clinical and/or eliminated through the body's usual tumor surveillance system. Clearly it would be advantageous to have targeted specific agents to treat and ultimately prevent breast cancer.

The story of SERM recognition and development [21, 22] has its origins in the study of tamoxifen (ICI 46,474) a drug originally discovered at the laboratories of ICI Pharmaceuticals Division, UK, in their fertility control program [23] as a potential post coital contraceptive. The drug failed in its primary application but slowly succeeded in a secondary application as a treatment for breast cancer [7, 24].

## 2 Tamoxifen, the first SERM

Tamoxifen is a pioneering medicine [7] because it became one of the first targeted treatments for cancer where the treatment strategy used today translated from the laboratory to clinical practice. The pharmacology of tamoxifen was studied extensively in animal models of mammary carcinogenesis to explore appropriate strategies to enhance disease control in patients. Tamoxifen was found to inhibit binding of estrogen to the ER mammary carcinomas both *in vitro* and *in vivo* [25–27]. *In vitro*, tamoxifen was demonstrated to have low affinity for the estrogen receptor [28], however, tamoxifen acts as a prodrug and is rapidly converted in the liver to a metabolite with high affinity to block the ER [29]. Tamoxifen, as well as its active metabolites, achieve stable, steady-state levels within the serum that remain constant during treatment ranging from months to years (over 7 years) [30].

An examination of tamoxifen treatment during the early stages of tumorigenesis in the rat mammary carcinoma model demonstrated that longer rather than shorter durations of tamoxifen would be necessary to use as a strategy for the adjuvant treatment of breast cancer [31–33] However, there was initial concern that long-term adjuvant tamoxifen would cause premature drug resistance. Nevertheless, clinical trail strategies eventually explored the optimal duration for tamoxifen therapy. It is now possible to assess the value of the idea of targeting tamoxifen to treat

women with ER positive tumors with long-term therapy. The Oxford Overview Analysis has established treatment trends based on the results from worldwide randomized clinical trials.

When the Overview analyses were first initiated, tamoxifen was the only universally used antihormonal agent. With no other competition, tamoxifen became the "gold standard" and established the principles of tumor targeting and identified the appropriate treatment strategy to aid survivorship [10, 34–36].

- Five years of adjuvant tamoxifen enhances disease free survival. There is a 50% decrease in recurrences observed in ER positive patients 15 years after diagnosis.
- Five years of adjuvant tamoxifen enhances survival with a decrease in mortality 15 years after diagnosis.
- Adjuvant tamoxifen does not provide an increase in disease free or overall survival in ER negative breast cancer.
- Five years of adjuvant tamoxifen alone is effective in premenopausal women with ER positive breast cancer; tamoxifen is ineffective in ER negative breast cancer.
- The benefits of tamoxifen in lives saved from breast cancer, far outweighs concerns about an increased incidence of endometrial cancer in postmenopausal women.

- Tamoxifen does not increase the incidence of second cancers other than endometrial cancer.
- No non-cancer related overall survival advantage is noted with tamoxifen when given as adjuvant therapy.

The Overview analysis process is now being applied to the numerous new aromatase inhibitors [6] that are being compared to tamoxifen directly, after a few years of tamoxifen or after a full five years of tamoxifen (Fig. 2). As a group, the aromatase inhibitors are superior to tamoxifen with improved overall survival and a reduced incidence of estrogen-like side effects.

Once antihormonal therapy had started to achieve optimal success in the treatment of node positive and node negative disease during the last decade, the trend for clinical research during the 1990s was to build on the successes of SERMs as treatments for disease so that breast incidence could be reduced in specific populations of women.

### 3 Tamoxifen and primary prevention

Early laboratory observations [37, 38] plus the finding that tamoxifen decreases contralateral breast cancer by 50% when the drug is used as an adjuvant therapy [39], made tamoxifen the agent of choice for evaluation as a chemo-preventive agent. A series of clinical trials aimed at primary

Fig. 2 A comparison of the strategies used to compare and contrast the therapeutic efficacy and side effects of various aromatase inhibitors with adjuvant tamoxifen in populations of postmenopausal women diagnosed with ER positive breast cancer



**Long Term Estrogen Deprivation Treatment**

AI = AROMATASE INHIBITOR

5 years tamoxifen

5 years AI

ATAC, *The Lancet* 2002, 359:2131-40
Howell et al, *The Lancet* 2005, 365:60-2
Thurlimann et al, *N Engl J Med* 2005, 353:2747-57

5 years tamoxifen

2-3 Tamoxifen | 3 years AI

Coombes et al, *N Engl J Med* 2004, 350:1081-92
Boccardo et al, *J Clin Oncol* 2005, 23:5138-47

5 years tamoxifen

5 years tamoxifen | 5 years AI

Goss et al, *N Engl J Med* 2003, 349:1793-802
Goss et al, *J Natl Cancer Inst* 2005,97:1262-71

breast cancer prevention established tamoxifen as the first drug to be approved for risk reduction of any cancer. The trials have been compared and contrasted [40] so only the conclusions will be considered after presenting the two main studies.

The National Surgical Adjuvant Breast and Bowel Project (NSABP) initiated the Breast Cancer Prevention Trial (P-1) in 1993 [11]. Approximately 13,000 pre and postmenopausal women were recruited because they were at high risk for developing breast cancer either due to age close to the peak incidence age of breast cancer, a high Gail score [41], or that had a history of lobular carcinoma *in situ*. The volunteers were randomized to receive placebo or 5 years of tamoxifen at the previously established daily dose of 20 mg/day. Tamoxifen produced a 49% (two-sided $p<0.0001$) decrease in the development of invasive breast cancers and a 50% (two-sided $p<0.002$) decrease in the development of non-invasive breast cancers. This effect was restricted to ER positive tumors (a 69% reduction), with no effect on the development of ER negative tumors [11]. The NSABP P-1 clinical trial was important in that it once again confirmed the requirement of the ER in a tumor for tamoxifen to be effective. The NSABP P-1 Trial, of all the prevention clinical trials, was the only one that did not incorporate the use of HRT in either of the trial arms. Allowing for the use of HRT in other prevention clinical trials may explain the blunted efficacy results when compared to the NSABP P-1 trial.

The International Breast Cancer Intervention Study (IBIS-I) was an international phase III chemoprevention trial comparing tamoxifen vs. placebo [42]. This trial enrolled approximately 7,000 pre-and post-menopausal women recruited on several continents. Their age was between 35–70 years prospectively determined to be at increased risk for breast cancer development [42]. Risk factors for breast cancer included at least a two-fold relative risk for patients ages 45–70 years, a four-fold relative risk for ages 40–44 and an approximately ten-fold relative risk for ages 35–39. Therefore, almost all participants (97%) had a family history of breast cancer. Approximately one-third of all patients used HRT while being treated on this clinical trial. At a median follow-up of 50 months, a 32% reduction in the development of breast cancers was documented (69 vs. 101, $p=0.01$). The risk reduction was demonstrated among the occurrence of both invasive (25% reduction, 64 vs. 85) and non-invasive breast cancers (69% reduction, 5 vs. 16), although these subset analyses did not achieve statistical significance. There was no reduction in the occurrence of ER negative breast cancers.

Taken together, the above data supports the conclusion that tamoxifen lowers the risk of developing ER-positive breast cancer in patients without a personal history of breast cancer, but that are at higher risk for the development of breast cancer due to genetic and/or other established risk factors. The prevention of breast cancer comes at the expense of well documented side effects, including an approximately 2–5 fold increase in uterine cancer [12, 42], and an approximately 2–3 fold increase in thromboembolic disease but only in postmenopausal women. In addition to increased menopausal symptoms, vaginal discharge and ocular abnormalities occur with tamoxifen These definitive clinical trial data suggest that chemoprevention with tamoxifen should focus on high risk premenopausal women [43]. It is anticipated, based on the Overview Analysis [36] 5 years of treatment will be followed by continuing protection for the following 10 years. Naturally, once tamoxifen treatment is stopped, menopausal symptoms will stop but the problem is whether women will wish to tolerate 5 years of tamoxifen. Solutions to the problem of compliance have focused on the selective serotonin reuptake inhibitors (SSRIs) (Fig. 3) but recent studies of the metabolism of tamoxifen have revealed important lessons that can potentially refine current chemoprevention strategies.

## 4 Refining treatment and prevention with tamoxifen

Alterations in the cytochrome P450 system impact upon tamoxifen metabolism and its efficacy. Tamoxifen metabolites have been recognized to have antiestrogenic activity [44, 45]. More recently, the cytochrome P450 2D6 (CYP2D6) metabolic pathway was shown to be important in the production of the tamoxifen metabolite, 4-hydroxy-N-desmethyl-tamoxifen (endoxifen (Fig. 4)). Endoxifen has similar potency to 4-hydroxy tamoxifen [46], but an approximately ten-fold higher circulating concentration than 4-hydroxy-tamoxifen [45]. Therefore, if cytochrome CYP2D6 is metabolically inactivated due to genetic variants of this particular phenotype or through inhibition of the 2D6 enzyme from use of concomitant medications that inhibit CYP2D6, tamoxifen cannot be metabolized to its active metabolites, resulting in diminished efficacy. Jin et al. [47] examined plasma endoxifen concentrations in healthy women 4 months of beginning adjuvant tamoxifen therapy. Endoxifen concentrations in the blood were found to be statistically significantly lower in patients with a CYP2D6 homozygous or heterozygous variant genotype when compared to homozygous wild-type genotype. Similarly low concentrations of endoxifen were also identified within this same cohort of patients among subjects using concomitant potent inhibitors of CYP2D6 such as paroxetine (Fig. 3). Such diminished endoxifen levels have recently been demonstrated to correlate with worse clinical outcome [48]. SSRIs are commonly prescribed to women taking tamoxifen for the treatment of associated hot flushes but the SSRIs range from potent to mild inhibitors of the

**Fig. 3** The classification of se
lective serotonin reuptake inhib
itors (*SSRIs*) used for the relief
of hot flashes in women being
treated with tamoxifen. The
SSRIs have high, intermediate
or low affinity for the CYP2D6
gene that metabolizes tamoxifen
or N desmethyl tamoxifen to 4
hydroxytamoxifen or endoxifen
respectively (See Fig. 4)



**HIGH**

**Fluoxetine**

**Paroxetine**

**INTERMEDIATE**

**Citalopram**

**Sertraline**

**LOW**

**Venlafaxine**
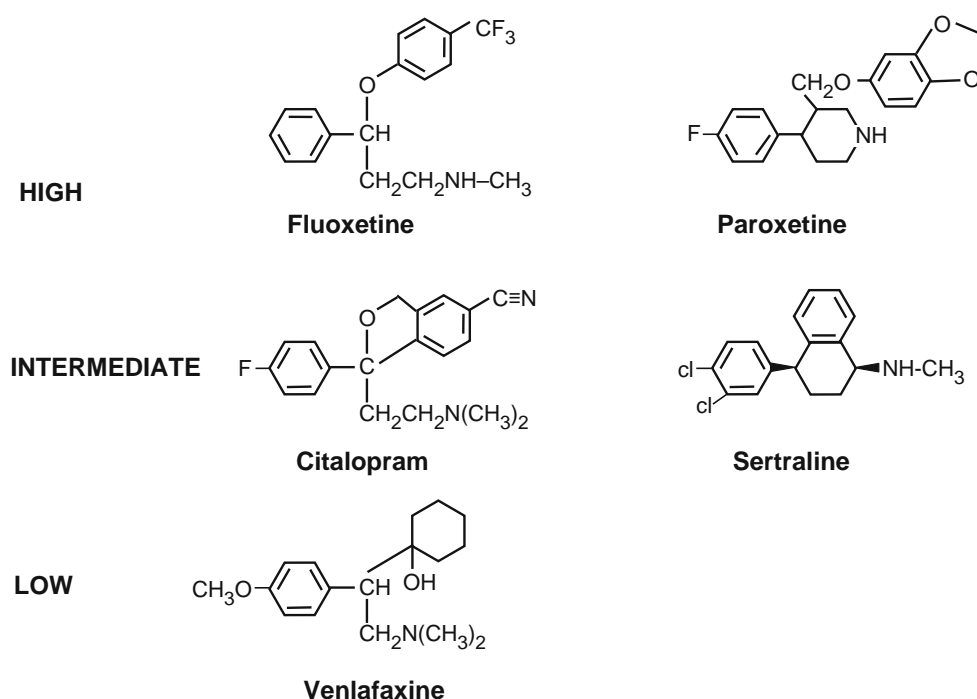
CYP2D6 cytochrome enzymes (Fig. 3). To determine
whether this knowledge has clinical relevance, a retrospec-
tive analysis was performed on a North Central Cancer
Treatment Group (NCCTG) randomized phase III clinical
trial [48]. In this trial, postmenopausal women with ER-
positive breast cancer were originally randomized to
adjuvant treatment with either tamoxifen for 5 years or
tamoxifen for 5 years followed by an additional year of
fluoxymestrone (NCCTG 89-30-52). Paraffin embedded
tumor samples from the tamoxifen only arm were geno-
typed for CYP2D6 wildtype and polymorphisms. Addi-
tionally, utilizing chart review, use of SSRIs was also
evaluated with respect to relapse-free survival (RFS),
disease-free survival (DFS) and overall survival (OS). In a
multivariate analysis, patients homozygous for CYP2D6
variant (CYP2D6*4/*4) trended towards worse RFS (HR,
1.85; $P=0.176$) and DFS (HR, 1.86; $P=0.089$), without
affecting OS (HR 1.12; $P=0.780$) compared to patients
heterozygous for the CYP2D6 variant (CYP2D6 *4/4) or
had wild-type CYP2D6 (CYP2D6 4/4). Additionally, the
symptoms of moderate and severe hot flashes segregated
with patients who were found to have the CYP2D6 *4/*4
homozygous gene polymorphism [48].When these data
(NCCTG 89-30-52) were re-analyzed to include evaluation
of concomitant CYP2D6 inhibitor use, multivariate analysis
revealed that patients with significantly decreased tamoxi-
fen metabolism due to either homozygous CYP2D6 *4/*4
variant genotype or due to concomitant use of an extensive
CYP2D6 inhibitor, had a statistically significantly worse
RFS (HR, adj=1.71, $p=0.017$) with a statistically signifi-
cant risk of breast cancer relapse (HR 3.12, $p=0.007$) [49].

This suggests that in order to individualize therapy for
premenopausal women with ER-positive early stage breast
cancers, tamoxifen might be best for patients homozygous
wildtype for CYP2D6 genotype and for those not requiring
SSRI's for the treatment of hot flashes. Alternatively,
Venlafaxine, which has low interaction with CYP2D6,
could be used to control hot flashes. Alternative therapies
such as the newer aromatase inhibitors might be considered,
for example, for postmenopausal patients with diminished
endoxifen metabolism either due to CYP2D6 genotyping or
need for utilizing SSRIs for hot flush symptom management
[50].

## 5 Recognition of selective estrogen receptor modulations

The recognition of SERM action and the realization that
nonsteroidal antiestrogens were, in fact, target site specific
estrogens and antiestrogens arose from the pharmacological
evaluation of tamoxifen during the transition from breast
cancer treatment to chemoprevention in the mid 1980s. It
was reasoned that if estrogen was beneficial for maintaining
bone density in postmenopausal women then perhaps the
long-term administration of tamoxifen to women without
cancer might prevent breast cancer but accelerate the
development of osteoporosis. However, the finding that
tamoxifen and the related compound raloxifene (then
known as keoxifene) would prevent bone loss in ovariec-
tomized rats [51–53] at doses that would prevent rat
mammary carcinogenesis [32, 54] changed that perspective.
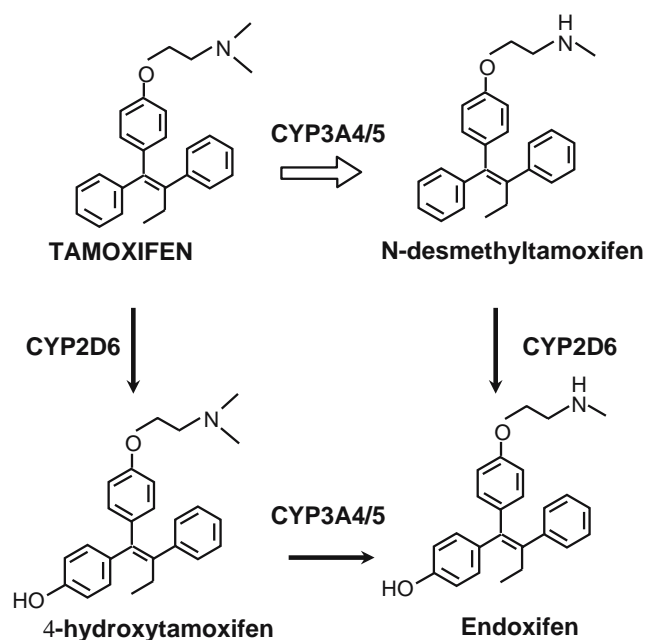More importantly, the simultaneous findings that tamoxifen

Fig. 4 The principal metabolites of tamoxifen observed in the serum of women receiving adjuvant tamoxifen therapy. Demethylation occurs through CYPA3/4 and aromatic hydroxylation through CYP2D6. The SSRIs (Fig. 3) block the metabolic activation of tamoxifen by binding to CYP2D6

could prevent estrogen-stimulated breast cancer growth but, at the same time, enhance the growth of the uterus or endometrial cancer [55, 56] rapidly translated to clinical practice with the finding that postmenopausal patients being treated with tamoxifen had an increased risk of developing endometrial cancer [57, 58]. This translational research resulted in gynecologists becoming involved in cancer care and safety procedures were established to avoid the progression of endometrial carcinoma stimulated to grow by tamoxifen. It was also reasoned that SERMs had opposing action in the uterus and breast and this translated to patients, why not translate the possibility of using SERMs to prevent breast cancer by treating osteoporosis?

## 6 The concept

A plan to prevent breast cancer as a public health initiative was initially described at the First International Chemoprevention meeting in New York in 1987. It is reasonable to simply state the proposal, published from the 1987 meeting and subsequently refined and presented at the annual meeting of the American Association for Cancer Research in San Francisco in 1989.

"The majority of breast cancer occurs unexpectedly and from unknown origin. Great efforts are being focused upon the identification of a population of high risk women to test "chemopreventive" agents. But, are resources being used

less than optimally? An alternative would be to seize upon the developing clues provided by an extensive clinical investigation of available antiestrogens. Could analogs be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true then a majority of women in general would be treated for these conditions as soon as menopause occurred. Should the agent also retain antibreast tumor actions then it might be expected to act as a chemosuppressive on all developing breast cancers if these have an evolution from hormone dependent to hormone independent disease. A bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century [13]." The concept was refined by 1990 [14] "We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer." This concept is exactly what has been translated to clinical practice [59, 60]: use a SERM (raloxifene) to treat osteoporosis and reduce the incidence of breast cancer as a beneficial side effect.

## 7 The SERM concept into practice

The Multiple Outcomes of Raloxifene (MORE) clinical trial was a multicenter, randomized, placebo controlled clinical trial utilizing raloxifene or placebo for the prevention of osteoporosis as its primary endpoint [59, 61, 62]. One of the multiple outcomes evaluated in this clinical trial was the secondary endpoint of breast cancer incidence. Therefore, post-menopausal women who met the criteria for diagnosis of osteoporosis were randomized in a 2:1 ratio to treatment with either of two doses of raloxifene—60 or 120 mg, or placebo. This population was an older population as the mean age of participants was approximately 66 years of age with over 80% aged 60 or older. Approximately 12% of trial subjects reported a first-degree relative with breast cancer. Additionally, approximately 29% of women reported previous HRT use at baseline and approximately 12% of women used HRT while being treated. In this population, raloxifene use was associated

with a 72% reduction in the incidence of invasive breast cancer (RR=0.28, 95% CI 0.17, 0.46) without significant impact on the incidence on *in situ* disease (nine vs. five cases for raloxifene and placebo, respectively, RR=0.90, 95% CI=0.30, 2.69). Of note, raloxifene had not effect upon the incidence of invasive estrogen receptor negative tumors (RR 1.13, 95% CI 0.35, 3.66).

In the Continuing Outcomes Relevant to Evista (CORE) trials, the chemopreventive effect of raloxifene were substantiated. This trial was essentially an extension of the MORE trial above for an additional 4 years of evaluation of the effect of extended raloxifene therapy [60]. Patients initially assigned to either 60 or 120 mg of treatment with raloxifene after the 4 years of the MORE trial were offered to continue raloxifene therapy with 60 mg of raloxifene (with the exception of patients still enrolled in the CORE trial assigned to 120 mg of raloxifene, i.e. less than 4 years of treatment). Similarly, patients initially assigned to the placebo arm of the MORE trial were continued on placebo. During the additional 4 years of evaluation, the continued use of raloxifene was associated with an approximately 59% reduction in the incidence of invasive breast cancer when compared to placebo (HR= 0.41, 95% CI −0.24–0.71) and a 66% reduction in the incidence of ER positive breast cancers (HR=0.34, 95% CI=0.18–0.66). Again, no protective effect was demonstrated in the development of ER negative breast cancers or *in situ* breast cancer. Over the 8 year period of evaluation from both the MORE data as well as the CORE data, raloxifene was demonstrated to reduce newly diagnosed invasive breast cancers by approximately 66% in total, when compared to placebo (HR=0.34, 95% CI= 0.22–0.50). This translated into an approximately 76% reduction in the relative occurrence of ER positive breast cancers (HR=0.24, 95% CI −0.15) with no resulting effect on ER negative breast cancers and *in situ* breast cancers, essentially providing confirmation of the earlier MORE trial results.

Based on analysis from the MORE trial evaluating cardiovascular risk, the Raloxifene Use for The Heart (Ruth) trial was undertaken with prevention of cardiac events and incidence of new breast cancer diagnosis as the primary objectives [63]. Approximately, 10,000 post-menopausal women with diagnosed coronary heart disease (CHD) or who were determined to be at risk for the development of CHD due to known risk factors such as diabetes mellitus, tobacco smoking and hypertension were randomized to treatment with either raloxifene 60 mg or placebo. Although raloxifene demonstrated no significant benefit for preventing primary coronary events in this patient population, (HR=0.95, 95% CI=0.84–1.07), a reduction in the development of invasive breast cancer was demonstrated. Once again, raloxifene use of approxi-

mately 5 years was associated with a 44% reduction (HR= 0.56, 95% CI=0.38–0.83) in the incidence of invasive breast cancer with treatment effect limited to ER positive breast cancers only. It is worthy to note that in this trial, analysis of breast cancer risk was performed and the preventative effect of raloxifene was also limited to patients at higher risk for developing breast cancer with a Gail score of 1.66 or higher. Most importantly, there was no increase in the risk of endometrial cancer confirming preclinical reports that raloxifene was substantially less effective than tamoxifen at stimulating endometrial cancer growth [64]. The final evaluation of raloxifene that will be presented is the chemoprevention of breast cancer growth determined in high risk postmenopausal women. The comparator medicine was tamoxifen.

## 8 Raloxifene and primary prevention

Patients were recruited into the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) trial from July 1, 1999 through November 4, 2004 [65]. This clinical trial randomizing patients to treatment with either tamoxifen or raloxifene for the primary prevention of breast cancer enrolled postmenopausal patients between the ages 35 and older, deemed to be at higher risk for the development of a first invasive breast cancer (the study primary endpoint) with either a 5 year predicted breast cancer risk of 1.66% based on the Gail model, or a previous history of lobular carcinoma *in situ* (LCIS) treated by local excision alone. It is worth noting that 19% of participants reported a family history of breast cancer in two or more first-degree relatives, and more than 71% reported a history of invasive breast cancer in one or more first-degree relative. Therefore, the mean predicted 5-year risk of developing breast cancer among the study population was 4.03% (SD, 2.17%). The primary endpoint of this randomized, double-blinded trial was the development of a first invasive breast cancer. Secondary endpoints also prospectively analyzed include, *in situ* breast cancer, endometrial cancer, all other cancers, cardiovascular disease, stroke, pulmonary embolism, DVT, transient ischemic attack, osteoporotic fracture, cataracts, death, and quality of life. The data was reported at a median follow-up time of 3.9 years. Both raloxifene and tamoxifen were equally effective at preventing the development of a first invasive breast cancer (RR 1.02; 95% CI, 0.82–1.28, p= 0.96). However, although not statistically significant, tamoxifen was better at preventing the occurrence of *in situ* breast cancers (57 vs. 80 for tamoxifen and raloxifene, respectively, p=0.052). This result is somewhat curious since the same mechanisms that would prevent an invasive breast cancer from developing could be expected to prevent

## New Breast Cancers
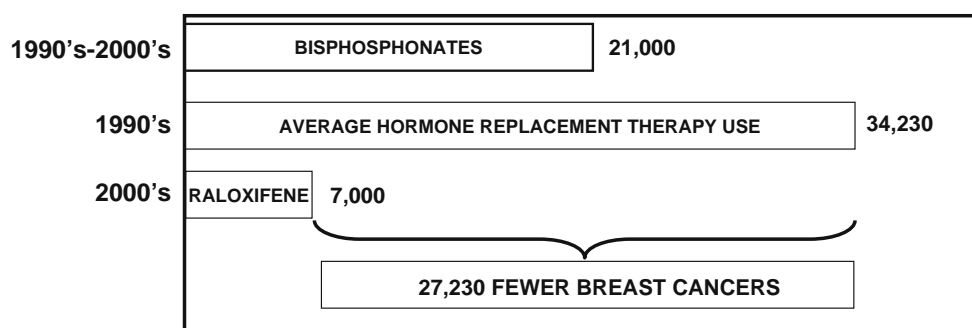


**Fig. 5** An estimation of breast cancer incidence in a population of 500,000 postmenopausal women with the same risk for osteoporotic fractures as participants in the CORE trial [60] treated for a 10 year period with a bisphosphonate, hormone replacement therapy (HRT) based on the average breast cancer risk between the Women's Health Initiative [17] and the Million Women's Study [18] or currently with raloxifene. The overall change in prescribing practices from the former practice of using HRT to prevent osteoporosis as the standard treatment to the current practice of prescribing raloxifene would be anticipated to produce a net decrease of 27,230 breast cancers. (Reprinted with permission from the European Journal of Cancer [43])

*in situ* breast cancers. However, this finding has been reported previously in both the MORE and CORE studies where raloxifene did not appear to reduce the risk of non-invasive breast cancers, although both studies had small numbers of total events. This trial confirmed that raloxifene was less stimulatory for the uterus with less uterine hyperplasia (RR, 0.16; 95% CI, 0.09–0.29) and although there were more reported cases of uterine cancer with tamoxifen (36 vs. 23 cases), this did not reach statistical significance (RR. 0.62; 95% CI, 0.35–1.08). Higher rates of thromboembolic disease were reported for tamoxifen with 30% less events occurring in the raloxifene treated subjects

**Fig. 6** A comparison of the structure of raloxifene with newer SERMs under development for the prevention of osteoporosis but with the potential to reduce the incidence of breast cancer as a beneficial side effect. Arzoxifene has a longer biological half life than raloxifene. Basedoxifene [74] and lasofoxifene [75] are two SERMs completing evaluation for the treatment of osteoporosis with the expectation that breast cancer incidence will be reduced
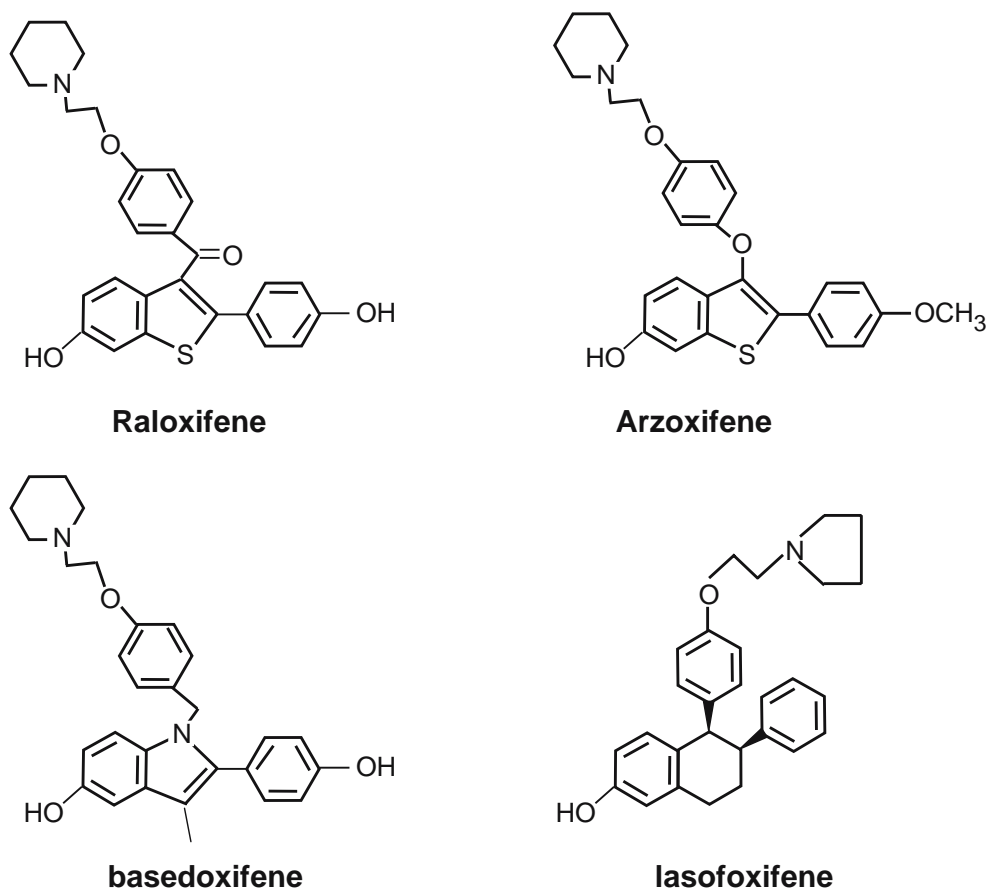
(RR, 0.70; 95% CI, 0.54–0.91). Additionally, higher rates of both cataract development ($p=0.002$) and patients undergoing cataract surgery ($p=0.03$) were higher in the tamoxifen arms. No difference in the rates of cardiovascular disease endpoints were reported. Interestingly, numerically there were higher numbers of unrelated cancers reported in the raloxifene arm. However, the overall numbers were small and the confidence intervals were wide suggesting that chance cannot be excluded as a possible cause. This clinical trial has now provided clinicians and post-menopausal patients with two viable options for primary prevention of breast cancer.

## 9 Direct and indirect approaches to chemoprevention

SERMs have proved to be valuable chemopreventive therapies to reduce the risk of breast cancer in both premenopausal (tamoxifen) and postmenopausal (tamoxifen and raloxifene) high risk women [66]. The approach to prevent the development of disease can be described as the direct approach for breast cancer chemoprevention. However, the changing fashion in restricting the application of HRT because of the definitive evidence that HRT increases the global incidence of breast cancer [18], and a decrease in HRT users will undoubtedly result in a fall in the incidence of breast cancer. If the availability of raloxifene to substitute for HRT for the prevention of osteoporosis is added into the equation, causing a reduction in breast cancer risk, then the SERMs will have gone some way in advancing the goal of reducing breast cancer incidence and mortality. The hypothetical benefits of the progress made in the past two decades in the chemoprevention of breast cancer are shown in Fig. 5. However, raloxifene is not an optimal drug for the prevention of breast cancer and osteoporosis. There are problems with both drug absorption and rapid Phase II metabolism [67]. In response, newer SERMS are now positioned (Fig. 6) to complete testing for the prevention of osteoporosis [68] and it is anticipated that they will also be a reduction in breast cancer incidence.

In closing, it is perhaps pertinent to state the current changes in the options for women's health that have occurred with the introduction of SERMs. Two decades ago, the concept [13] that SERMs could be useful multi-functional medicines has now become a clinically validated reality. During the past decade, there have been important changes in the evolution of ideas about women's health. HRT does not provide an easy solution to prevent coronary heart disease, osteoporosis and Alzheimer's disease. The WHI [17, 69–72] and the Million Women's Study [18] have defined the price to be paid with no decreases in coronary heart disease in the elderly, increases in breast cancer and modest but significant increases in Alzheimer's disease. There are suitable alternatives to the prevention of osteoporosis using bisphosphonates [73] but this intervention does not affect breast cancer or coronary heart disease. Statins have proven to be effective in retarding the development of arteriosclerosis and coronary heart disease. There is, however, no firm prospective evidence that these medicines reduce the incidence of breast cancer. In contrast, SERMs such as raloxifene can reduce the risk of osteoporosis and breast cancer. Admittedly raloxifene did not fulfill the promise to reduce the risk of coronary heart disease in the Raloxifene use for the Heart (RUTH) trial [63] but it is fair to say that the menu of medicines now available to prevent diseases that develop after menopause have steadily improved the prospects retarding disease development over the past 20 years.

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# EDITORIAL

# The Rise of Raloxifene and the Fall of Invasive Breast Cancer

V. Craig Jordan

Bernard Fisher has recently stated, "A clinical trial is just a mechanism by which to evaluate what you have done in the laboratory" (*Oncology News International*, March 2008). In this issue of the Journal, Grady et al. (1) have analyzed the incidence of invasive breast cancer in a clinical trial of women treated with raloxifene with the intention of reducing their risk of dying from coronary heart disease. To the casual observer, an analysis of this nature would seem to be unusual, if not a bit bizarre, but the fact is that raloxifene is a selective estrogen receptor modulator (SERM) that has estrogen-like activity to reduce low-density lipid (LDL) cholesterol (2) and to reduce the risk of fractures in osteoporosis (3), and antiestrogenic properties to block the growth of breast cancers (4). When the Raloxifene Use for the Heart (RUTH) trial started, raloxifene was approved for the prevention of osteoporosis in high-risk postmenopausal women, and it was known from clinical trials that raloxifene produced a decrease in invasive breast cancer (5). So where did all the ideas come from to examine these qualities of raloxifene, which had previously failed its original application as a breast cancer drug (6)? The answer is the laboratory.

Raloxifene started life in the laboratories at Eli Lilly as Y156758, a nonsteroidal antiestrogen (7) with a high affinity for the estrogen receptor (ER) (8) and a primary application as a treatment for breast cancer. Regrettably, this polyhydroxylated class of drugs has a very short biological half-life (9) and subsequent clinical studies with the drug under the name keoxifene also showed virtually no activity in patients who had failed tamoxifen treatment (10). Further development as a breast cancer therapy was abandoned in the late 1980s. However, at this time, selective ER modulation was recognized (11–13) for "nonsteroidal antiestrogens" (tamoxifen and raloxifene are members of this class) and a new opportunity occurred for clinical development (14). This opportunity was based on the laboratory finding that tamoxifen and keoxifene (aka raloxifene) simultaneously maintained bone density in ovariectomized rats (12) and inhibited rat mammary carcinogenesis (15). These findings rapidly translated into the hypothesis that perhaps one could reduce the risk of breast cancer by treating women with a drug that maintained bone density, thereby reducing the risk of osteoporosis. It was well known that this class of drugs lowered circulating cholesterol in laboratory animals; in fact, tamoxifen had a patent as a potential hypocholesteremic agent since the 1960s (16,17). Based on all of these laboratory data, a clinical development strategy was simply stated that was to eventually resurrect and catalyze the rise of raloxifene (18):

> Nevertheless there is a real concern about being able to target the right population [for prevention]. We cannot predict who will develop breast cancer; we can only guess at the probability. Furthermore "high risk" women are, in fact, only a minority of those who will develop breast

cancer so any success must be balanced against as yet unknown accumulative toxicities. Is this the end of the possible applications for antiestrogens? Certainly not. We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer.

In the late 1980s and early 1990s, clinical studies were exploring the pharmacology of tamoxifen as a prelude to its use in high-risk women as a potential chemopreventive agent. What was found was that tamoxifen lowered LDL cholesterol in postmenopausal women but did not affect high-density lipid cholesterol (19,20). More importantly, tamoxifen enhanced spinal bone density compared with placebo in a randomized clinical study of postmenopausal women (21). It was about this time that scientists at Eli Lilly confirmed (22) the findings of the earlier laboratory studies that raloxifene had potential for maintaining bone density (12) and also lowered circulating cholesterol. The scene was therefore set to test raloxifene as a SERM to prevent fractures from osteoporosis in the Multiple Outcomes of Raloxifene Evaluation (MORE) trial (1994) and, subsequently, to initiate the RUTH trial (1998). Both trials naturally evaluated the original hypothesis that multiple diseases could potentially be controlled with a SERM, thereby enhancing public health (14,18). It is now clear based on clinical trials data, however, that raloxifene is not effective to reduce the risk of coronary heart disease (23,24). It could be that patients recruited to the RUTH trial have disease that is too far advanced for the modest reductions in LDL cholesterol to have any impact on pathology. The SERM approach may work only in patients who have very early atherosclerotic lesions so that long-term therapy can effectively retard the development of pathology. In the years to come, it may be impossible to answer this question by examining populations of women who are using raloxifene to prevent osteoporosis because of the widespread use of statins to reduce LDL cholesterol.

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One interesting aspect of the study of Grady et al. (1) is the 44% reduction in invasive breast cancer, which also comprises a 55% reduction in invasive ER-positive breast cancer. This placebo-controlled study can be compared with the Study of Tamoxifen and Raloxifene (STAR), where raloxifene was noted to be equivalent to tamoxifen at reducing the risk of breast cancer (25). Although the STAR, was not placebo controlled, in Fisher's pioneering placebo-controlled tamoxifen study, the National Surgical Adjuvant Breast and Bowel Project P-1 trial, there was a 50% reduction in invasive breast cancer and a 69% reduction in ER-positive breast cancer (26,27). Overall, these data contrast with the MORE trial, in which there was a 76% decrease in invasive breast cancer and 90% decrease in ER-positive breast cancer. The question is why? One plausible explanation for the greater reduction in invasive breast cancer in the MORE trial than in the RUTH and STAR trials could be the low circulating levels of estradiol in postmenopausal women at risk for osteoporosis compared with those in women in both the RUTH and STAR trials. The polyphenolic compounds related to raloxifene are competitive inhibitors of estrogen action, and it is also known that raloxifene has only a 2% bioavailability, with rapid excretion (28). Once patients become noncompliant about taking raloxifene, there would be no protection for the development of invasive breast cancer. Although the numbers are very small in the study of Grady et al. (1) and the MORE trial (5), raloxifene appears to be poor at controlling the risk of developing noninvasive carcinomas. Indeed, tamoxifen seems to be marginally superior to raloxifene in controlling noninvasive breast cancer in the STAR trial (25).

Overall, clinical evidence is accumulating that the SERMs hold great promise in being able to control multiple diseases (29). This is the good news because, until recently, it was generally believed that hormone replacement therapy was the answer to controlling the development of coronary heart disease and osteoporosis, but at the price of an enhanced risk of invasive breast cancer (30,31). For the future, this is no longer acceptable and the SERMs may be one way of further advancing targeted public health.

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Preface
# Preface: controversies in breast cancer
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On 3–4 September 2007 a faculty of world experts on breast cancer gathered in the Royal College of Physicians, Edinburgh, not to present their latest results, but to debate a series of controversial issues relating to breast cancer with an invited audience.

The intention was to highlight the points that made the topics controversial, with the initial objective being to clarify rather than resolve the issues. In this way additional thought, perspective and understanding would be forthcoming and ultimately resolution might be more likely. This volume represents a summary of the proceedings as formulated by each of the presenters.

Before delving into the communications, the reader should appreciate the nature of each session and the brief given to the faculty. Topics and faculty were selected by the chairman, and each chairperson has been invited to provide a foreword for their sessions. It is also important to have a perception of the mechanism by which the controversies were tackled. Thus, in certain cases, most notably in the presentations on psychosocial aspects of breast cancer, one facet of the topic was put forward by a proposer and a different, often opposing, view by another member of the faculty. To achieve a balance, it may therefore be necessary to integrate several individual short communications. Furthermore, in the sessions on challenging established dogma, the faculty members were asked to be a devil's advocate and to be provocative in their presentations, providing that they could reference substantiating results. It should therefore not be assumed that the perspectives are necessarily firmly held views of the authors.

The short communications are aimed at provoking thought in the hope of progressing science and improving practice. The volume should not be regarded as a standard text reflecting routine proceedings; instead, it is intended to be a stimulating text, challenging the reader to question perceived wisdom.

## Acknowledgement

Short communication

# Oestrogen is bad for patients with breast cancer?

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Breast cancer is a hormone-dependent disease, and a proportion of patients with oestrogen receptors (ERs) will respond to ovarian ablation [1-3]. For this reason, oestrogen is considered bad for patients with breast cancer. This short communication presents our evolving understanding of oestrogen's role as a survival signal in breast cancer and new emerging knowledge of the apoptotic actions of oestrogen [4].

Synthetic oestrogens based either on the structure of triphenylethylene or the very potent but shorter acting diethylstilboestrol [5,6] were described more than 60 years ago. This proved to be a cheap source of new medicines. High-dose synthetic oestrogen administration was found to be effective in the treatment of breast and prostate cancer [7], but low-dose synthetic oestrogens never really became accepted as hormone replacement therapy in postmeno-pausal women. Indeed, diethylstilboestrol subsequently achieved notoriety as an oestrogen supplement to prevent recurrent abortion. Children of treated mothers had a high incidence of clear cell carcinoma of the vagina [8,9]. In contrast, the synthetic oestrogens based on triphenyl-ethylenes were subsequently to undergo a metamorphosis and be transformed into anti-oestrogens used for the treatment of breast cancer [10].

Based on the link identified between oestrogen and the development and growth of some breast cancers, the current strategy for the treatment and prevention of ER-positive breast cancer is the application of long-term antihormonal therapy [11]. The use of long-term tamoxifen therapy [12] has had a profound effect on survival, but in addition the wide distribution of tamoxifen has resulted in a declining death rate from breast cancer over the past few years. Currently, the aromatase inhibitors [13-15] are proving to represent a modest improvement over tamoxifen therapy, especially for the postmenopausal woman with concerns about endometrial cancer and blood clots. However, tamoxifen remains the treatment of choice for the premenopausal woman with ER-positive breast cancer.

The past 30 years have seen dramatic advances in the practical prospects for the chemoprevention of breast cancer. Studies in the laboratory with tamoxifen [16,17] and raloxifene [18,19] have now translated into clinical practice for either chemoprevention of breast cancer in high-risk women with tamoxifen [20,21] or treatment of osteoporosis with prevention of breast cancer with raloxifene [22,23]. However, widespread use of long-term antihormonal therapies for the treatment and prevention of breast cancer creates consequences for the tumour in the form of antihormonal drug resistance. Never-theless, laboratory study of antihormonal drug resistance has revealed an unanticipated vulnerability of breast cancer cells.

It has been known for about 20 years that long-term oestro-gen treatment of athymic mice inoculated with the ER-positive breast cancer cell line MCF7 will result in trans-plantable ER-positive tumours [24]. Tamoxifen will initially prevent tumour growth, but long-term tamoxifen therapy causes tumours to become drug-resistant, which is expres-sed as tamoxifen-stimulated growth [25]. This model system replicates the clinical situation for the treatment of advanced breast cancer, and second-line therapies in the clinic are usually an aromatase inhibitor or the pure anti-oestrogen fulvestrant [26,27]. However, the process of developing tamoxifen-stimulated tumour growth in the laboratory, which takes 1 to 2 years, does not replicate adjuvant therapy with tamoxifen, which has a duration of 5 years. To address this issue, tamoxifen-stimulated tumours were serially trans-planted into successive generations of athymic mice and a novel form of drug resistance was recognized. Tamoxifen and other selective ER modulators (SERMs) such as raloxifene stimulate tumour growth [28], but remarkably oestrogen now does not support tumour growth but causes rapid tumour regression [29,30].

**Figure 1**
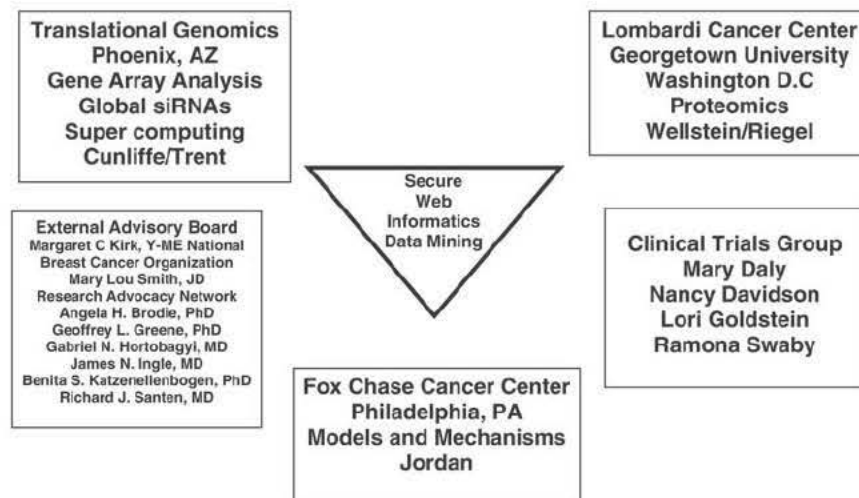


Organization of Department of Defense Center of Excellence Grant. Shown is the organization of our Department of Defense Center of Excellence Grant entitled 'A new therapeutic paradigm for breast cancer exploiting low-dose oestrogen-induced apoptosis'. The model systems to study the survival and apoptosis induced with oestrogen are being used in time course experiments at the Fox Chase Cancer Center. The materials are distributed to Translational Genomics for genomic analysis using comparative genomic hybridization, small interfering (si)RNA analysis or Agilent gene array analysis, and the Vincent T Lombardi Cancer Center is involved in conducting proteomics analysis. All results are uploaded into a shared secure web for data processing and target identification by our informatics and biostatistical group. Each laboratory is able to validate emerging pathways and study individual genes of interest. Our programme is integrated with a clinical trials programme that provides patient samples for validation of apoptotic or survival pathways. We are grateful to our external advisory board of patient advocates and professional colleagues for their continuing advice and support.

This action of oestrogen after 5 or more years of tamoxifen therapy demonstrates that there is an evolution of drug resistance in breast cancer cells. This was recently classified [31]. The early phases of drug resistance with tamoxifen are referred to as phase I resistance. This is indicated by a tumour growing with either tamoxifen or oestrogen treatment. In contrast, phase II resistant tumours grow only with tamoxifen, and oestrogen kills tumour cells. Similar studies are now being conducted using long-term oestrogen deprivation to replicate what will occur with the aromatase inhibitors [32].

Early studies growing MCF7 breast cancer cells in oestrogen-free media identified increased intracellular ER levels and spontaneous cell growth [33,34]. Several oestrogen-independent clones were isolated for study [35,36] and the idea was proposed that MCF7 cells are hypersensitized to grow in extremely low levels of oestrogen (below the level that can be detected or further reduced) [37]. However, Song and coworkers [38] observed that increasing concentrations of oestradiol could increase apoptosis in oestrogen-deprived cells by increasing the concentration of Fas ligand that activates death receptor pathways. Thus, the original observations that phase II tamoxifen resistant tumours could be treated with physiological oestrogen [29,30] were extended to aromatase inhibitor resistant cells. However, in contrast to the study

conducted by Song and coworkers [38], phase II tamoxifen resistant tumours respond to increasing oestrogen treatment by increasing the Fas receptor, and decreasing HER2/neu and nuclear factor-κ, which is associated with tumour regression [39]. Furthermore, MCF7 cells kept for many years under oestrogen-depleted conditions using medium containing stripped foetal bovine serum produce rapid apoptosis via an intrinsic mechanism directed at the mitochondrion [40,41]. However, both Lewis and coworkers [41] and Song and Santen [42] found that apoptosis is modulated through bcl-2 or bcl-2XL.

It is also perhaps important to note that the new knowledge about oestrogen action emerged through re-examination of existing cell lines. In early reports on the effects of oestrogen withdrawal, no oestrogen-induced apoptosis was noted [35,36], but by altering culture conditions or extending the period of oestrogen exposure, apoptosis occurs [40,41,43]. Overall, the phenomenon observed with long term oestrogen withdrawal is similar to the phase II resistance of the model described for SERMs [32].

Lonning and coworkers [44] addressed the hypothesis that patients with ER-positive breast cancers who have been treated exhaustively with antihormonal therapy could potentially respond to high-dose oestrogen therapy. Thirty-

## Figure 2
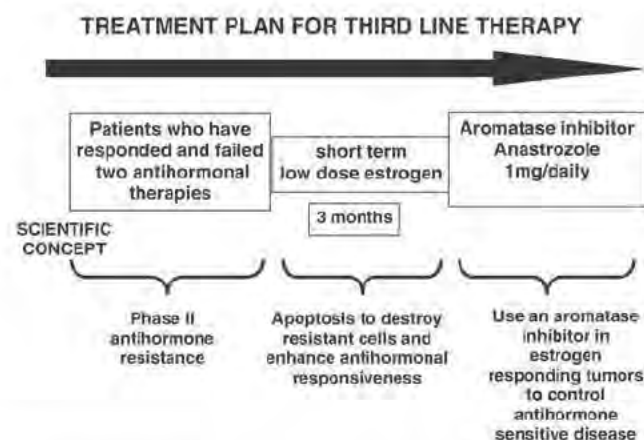


**TREATMENT PLAN FOR THIRD LINE THERAPY**

Anticipated treatment plan for third-line endocrine therapy. Patients must have responded and failed two successive antihormonal therapies to be eligible for a course of low-dose oestradiol therapy for 3 months. The anticipated response rate is 30% [44] and responding patients will be treated with anastrozole until relapse. Validation of the treatment plan via the Center of Excellence Grant (Figure 1) will establish a platform to enhance response rates with apoptotic oestrogen by integrating known inhibitors of tumour survival pathways into the 3-month low-dose oestrogen debulking treatment plan. The overall goal is to increase response rates and maintain patients for longer on antihormonal strategies before chemotherapy is required.

two patients with advanced breast cancer previously exposed to between two and ten (median four) endocrine treatments were treated with diethylstilboestrol 5 mg three times daily. Therapy was well tolerated but four patients terminated treatment within 2 weeks of starting and another two stopped treatment before progression. One of these patients had stable disease for 15 weeks and one a partial response for 39 weeks. Of the remainder, four patients obtained a complete response and six patients a partial response. Two patients had stable disease for 6 months and one for more than 1 year. Overall, these extremely encouraging preliminary studies with high-dose oestrogen therapy are complemented by anecdotal reports of the effectiveness of low-dose oestrogen treatment for those women with endocrine refractory breast cancer after exhaustive antihormonal therapy (Ingle J, Dixon M, personal communication). As a result, several clinical studies are currently underway (Ellis M, Santen R, personal communications).

Based on preclinical laboratory modelling, we have translated the new biology of oestrogen action into a Department of Defense Center of Excellence grant with laboratory and clinical collaborators illustrated in Figure 1. Our goal is to define the pathways for oestrogen-induced survival and apoptosis in endocrine responsive breast and endometrial cancer, and to use the emerging database to guide the interpretation and development of a series of clinical trials.

The ultimate goal of our clinical trial design is illustrated in Figure 2 and currently consists of two separate but interconnected therapeutic oestrogen studies, designed to determine the lowest dose of a 12-week course of oestrogen that causes a positive therapeutic effect.

In summary, the development and extensive clinical application of long-term antihormonal therapy [11] has had consequences for the patient with the development of antihormonal drug resistance in some breast cancers [31]. However, with the development of drug resistance to exhaustive antihormonal therapy, a vulnerability of the cancer has been exposed. The recognition of the new biology of oestrogen action that causes apoptosis in sensitive breast tumours now opens an unanticipated door of opportunity to exploit the findings to aid patients. Although the actual clinical responses may not be profound in unselected patient populations or in populations whose tumours do not have the correct (stage II) form of breast cancer drug resistance, our ability to decipher apoptotic mechanisms from laboratory models, and eventually to target patients appropriately, may have profound and positive effects for some patients. The translational knowledge gained over the coming few years may again provide unanticipated opportunities to exploit the discovery of 'apoptotic triggers' for other forms of cancer.

It is perhaps pertinent to restate that for 70 years there has been an 'ebb and flow' relationship for the role of oestrogen in breast tumour homeostasis. We have illustrated in this article many of the changing fashions that have occurred in the perception of oestrogen as either hero or villain with repect to women's health. The effects of modulating the ER system in the breast, at one time or another, have been dismissed because the effects are small or believed to be of no major consequence. Nevertheless, the number of events becomes accumulative. By way of example, it is important to recall that initial use of tamoxifen, a failed contraceptive, to treat unselected populations yielded only modest responses for some patients with metastatic breast cancer [45]. Years later, after deciphering the target populations and translating the appropriate treatment strategies from the laboratory to the clinic, the drug became the 'gold standard' for endocrine therapy [45] and was credited with improving the survival of hundreds of thousands of women [12]. The challenge for the future is to exploit the profound apoptotic action of oestradiol as a lead to develop innovative new therapies for cancer.

## Acknowledgements

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CLINICAL UPDATES IN

# Breast Cancer

ELSEVIER

# Welcome

In this latest issue of *Clinical Updates in Breast Cancer* we begin with highlights from the recent ASCO 2007 annual meeting which took place this year in Illinois'"windy city", Chicago.

We are also delighted and proud to feature three articles written by esteemed experts in their field. The first article is by Dr V. Craig Jordan and his colleagues who write on the current clinical indications for fulvestrant, covering topics from the structure and mechanism of action, dosing, and how fulvestrant should be used in sequence with other agents.

Associate Professor Peter Graham discusses the optimal timing of radiotherapy with chemotherapy and with hormonal therapy. He also reports on the STARS trial which is anticipated to open this year.

The third article, by Professor Christobel Saunders, reports on the very important topic of MRI screening of women in Australia who are at high risk of breast cancer.

Finally, we end the issue with a paper from the *International Journal of Radiation Oncology Biology Physics* which investigates whether breast radiotherapy is beneficial in women with favourable early breast cancer treated by lumpectomy plus tamoxifen or anastrozole.

Lilian Leung
Medical Editor

# Editorial Advisory Board

# The current clinical indications for fulvestrant

## An article by Roshani R. Patel, Jennifer R. Pyle and Dr V. Craig Jordan

**V. Craig Jordan** is the Vice President and Research Director for Medical Sciences at the Fox Chase Cancer Centre. He is the Alfred G. Knudson Chair of Cancer Research and Professor of Cancer Biology at the University of Pennsylvania, USA. Dr Jordan obtained a BSc (1969), PhD (1972) and DSc (1984) degrees in Pharmacology from the University of Leeds, UK. Dr Jordan has received worldwide recognition for his work on tamoxifen and raloxifene and most notably the Kettering Prize from the General Motors Cancer Research Foundation (2003), The Bristol Myers Squibb Award (2001), and The American Cancer Society Medal of Honor (2002). In 2002, Her Majesty, the Queen, appointed him as Officer of the Most Excellent Order of the British Empire (OBE) for his services to international breast cancer research.

*Dr V. Craig Jordan*

## Introduction

Fulvestrant is a novel treatment for postmenopausal women with advanced breast cancer who have previously failed tamoxifen therapy. It has been shown to be as effective as third-generation aromatase inhibitors in phase III trials.[1] The selective oestrogen receptor modulator (SERM) tamoxifen has antagonist actions in the breast but also agonist effects on other tissues including the endometrium. Such agonist activity has been shown to stimulate endometrial thickening and increase the risk of endometrial cancer.[2] Drug resistance to tamoxifen is often expressed as tamoxifen-stimulated growth via the oestrogen receptor (ER).[3] In contrast, fulvestrant is an ER antagonist that has no agonist effects.[1] Fulvestrant shows minimal side effects and is well tolerated.[4]

## Structure and mechanism of action

Faslodex™ is a long-acting formulation of fulvestrant used to treat hormone receptor positive metastatic cancer. Fulvestrant inhibits oestrogen-stimulated tumour growth by first binding to the ER[5] and then promoting destruction of the complex. Fulvestrant has a chemical structure similar to that of oestradiol but it also contains a strategically placed long alkylsulphinyl side chain (**Figure 1**) at the 7α position.

This structure allows fulvestrant to bind with a high affinity to the ER and thereby compete with oestradiol for ER interaction. Once the fulvestrant molecule binds to the ER, the shape of the complex is changed dramatically from the normal shape of the oestrogen-ER complex. A cascade of events then occurs, including the inhibition of activating function 1 and 2, inhibition of receptor dimerisation, and the inhibition of transcription co-activator recruitment.[6] Fulvestrant causes the ER to be tagged with ubiquitin, a small protein that latches onto damaged or mutated proteins, which results in the drug-bound ER being rapidly degraded by proteasomes (**Figure 2**).[7-9] Consequently, the cellular ER concentration is reduced thereby inhibiting the signal transduction pathway for tumour growth.

The novel mechanism of action for fulvestrant makes it a suitable agent to treat SERM-resistant advanced breast cancer.

## Dosing of fulvestrant

Fulvestrant (250 mg) is given as a monthly intramuscular injection. The drug is slowly absorbed by the body from the injection site. Pharmacokinetic studies have demonstrated that regardless of the dosing schedule, there is no difference in bioavailability or release of the drug from the injection site.[10] Further clinical studies indicate that once-monthly doses of fulvestrant 250 mg result in $C_{trough}$ values that double between the first and sixth doses. This results in a 3–6 month period before steady-state levels are achieved, due largely to the slow and sustained release of the drug.[11] As the pharmacokinetics of fulvestrant allow for once-a-month dosing, there may be benefits for patients in whom compliance with oral medication is an issue. As well as having to remember to take their medication, taking a pill each day can serve as a daily reminder of their condition. By receiving an injection once a month, compliance can be assured while the patient receives continued follow-up with their practitioners and psychosocial support from the care team.[12]

## Clinical application and positioning of fulvestrant in the endocrine sequence of therapy

Currently, the clinical use of fulvestrant primarily applies to postmenopausal women with advanced ER-positive breast cancer who have failed prior endocrine therapy or have recurrent breast cancer. To understand how fulvestrant should be used in sequence with other agents, it is important to understand the results from two large phase III trials in the second-line setting and some important phase II trials regarding third-line treatment. In addition, another study has investigated the role fulvestrant could play in the first-line treatment of advanced breast cancer.

Two large multicentre, randomised trials were run in parallel to
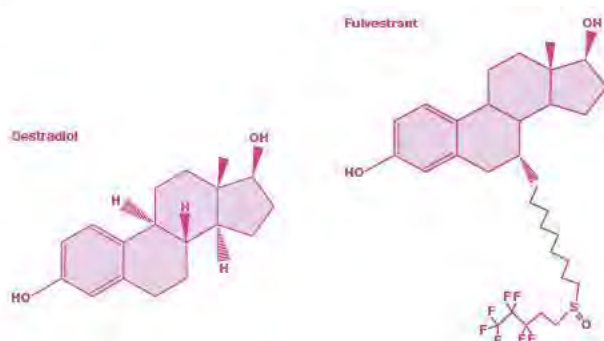


*Figure 1. The chemical structures of oestradiol and fulvestrant*
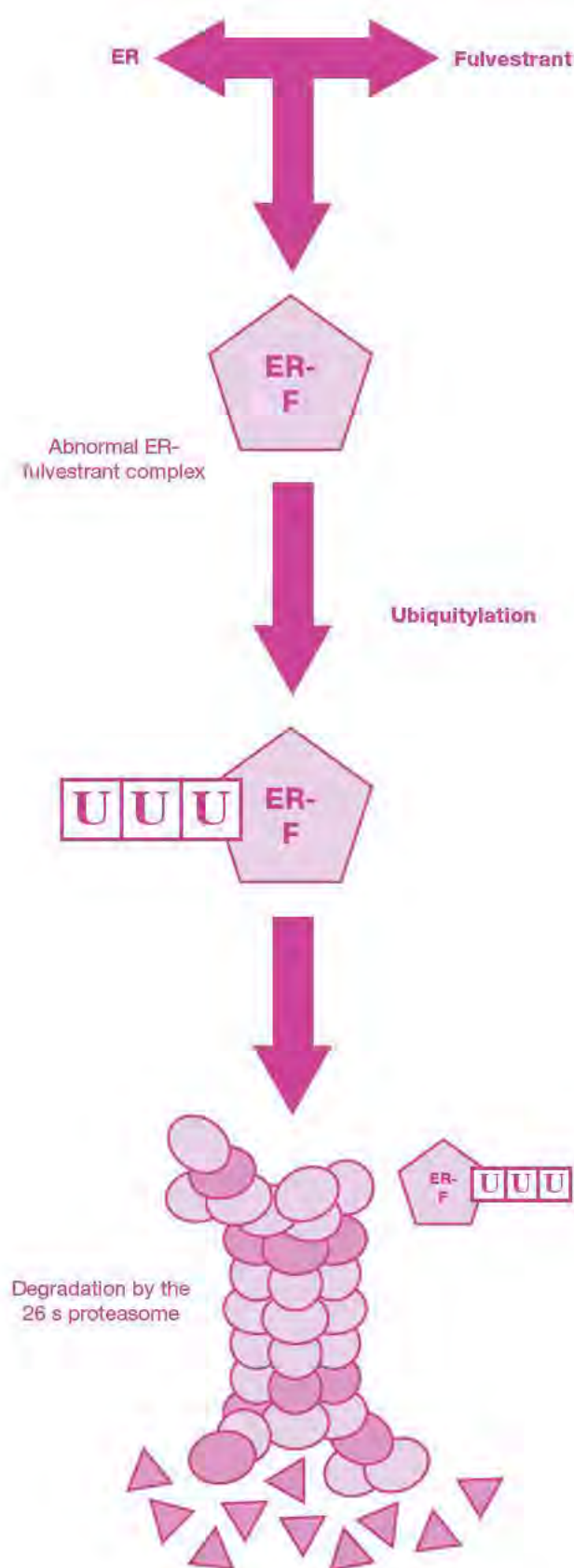
*Figure 2. The cascade of events leading to degradation of the oestrogen receptor, including binding of fulvestrant, conformational change of the oestrogen receptor, and degradation of the oestrogen receptor by proteasomes.*

determine whether the use of fulvestrant or anastrozole after disease progression on tamoxifen would achieve better outcomes. Trial 0020 involved Europe, Australia and South America, while trial 0021 was a North American study.

### Trial 0020

Trial 0020 recruited 451 patients. Fulvestrant was administered as a single 250 mg intramuscular dose in 222 patients. Anastrozole was administered orally as a 1 mg dose to the 229 patients in the other group. Patient demographics were similar in both groups and patients were followed for an average of 14 months. Overall, there was no statistically significant difference in clinical benefit between the two groups. In the arm receiving fulvestrant, 10 (4.5%) patients had a complete response, 36 (16.2%) patients had a partial response and 53 (23.9%) patients had stable disease for more than 24 weeks. The arm receiving anastrozole had four (1.7%) patients with a complete response, 32 (14%) patients with a partial response and 67 (29.3%) patients with stable disease for more than 24 weeks. The average time to progression was 5.5 months with fulvestrant and 5.1 months with anastrozole.[13]

### Trial 0021

The design of trial 0021 was very similar to trial 0020 except that fulvestrant was administered as two 125 mg injections (one in each buttock). There were 206 patients in the fulvestrant arm and 194 patients in the anastrozole arm. Average follow-up was 16.8 months. In the fulvestrant arm, 10 (4.9%) patients had a complete response, 26 (12.6%) had a partial response, and 51 (24.8%) had stable disease for more than 24 weeks. The arm receiving anastrozole had seven (3.6%) patients with a complete response, 27 (13.9%) with a partial response and 36 (18.6%) with stable disease for more than 24 weeks. On average, time to progression was 5.4 months with fulvestrant and 3.4 months with anastrazole.[4]

Both studies were combined for further analysis. As a second-line treatment, fulvestrant was similar to anastrazole in terms of overall survival. Further follow-up was obtained for patients who completely (20 in the fulvestrant group and 11 in the anastrazole group) or partially (62 in the fulvestrant group and 59 in the anastrazole group) responded to treatment. The duration of response was significantly longer in the fulvestrant group (16.7 months) compared with the anastrozole group (13.7 months). Both drugs were well tolerated and had similar side effect profiles that included hot flushes, gastrointestinal disturbances and thromboembolic disease. There were significantly more subjective complaints of joint pain in the anastrozole group compared to the fulvestrant group.[14]

Smaller clinical trials demonstrate that fulvestrant has promise after disease progression with aromatase inhibitors (AIs) in select patients. The first trial, the North Central Cancer Treatment Group Trial N0032, evaluated the use of fulvestrant in postmenopausal women who had disease progression of oestrogen receptor and/or progesterone receptor (ER/PR)-positive disease. Seventy-seven patients were previously treated with an AI or an AI plus one other antihormonal agent. Patients received anywhere from one to 10 cycles of treatment (average of two cycles because of disease progression). Of 21 patients who received only an AI in the past, six patients had a partial response and five patients had tumours that

did not progress during the 6 months they were followed. The remaining 56 patients received an AI and tamoxifen in the past. After subsequent treatment with fulvestrant in this group, 11 patients had tumours that did not progress for at least 6 months and five patients had a partial response. When evaluating the average disease progression of all 77 patients in the study, the clinical benefit rate (as defined by CR + PR + stable disease for at least 6 months) was 35.1% (90% CI, 26.0% to 45.0%).[15]

Another trial, known as SAKK 21/00, evaluated two groups of patients with advanced breast cancer. The first group (group A) initially had AI-responsive disease but progressed while on AIs (70 patients). The second group (group B) had AI-resistant disease and never responded to AIs (20 patients). All patients except two had been treated with an AI in the past. In group A, 84% had also received either tamoxifen or toremifene; in group B, 89% had received tamoxifen. Moreover, 36% of the patients in group A and 32% of those in group B had received prior chemotherapy. Patients in both groups received an average of four injections or 3.8 months of treatment with fulvestrant. In this study, only one patient in group A had a partial response. Of the remaining patients, 18 had stable disease for more than 24 weeks (11 for more than 36 weeks and six for more than a year). In group B, six patients had stable disease for more than 24 weeks, three patients had stable disease for more than 36 weeks, and one patient with metastatic lung disease had a complete response. The patient with a complete response was still on fulvestrant after 1 year.[16]

Finally, the question has been addressed as to whether fulvestrant is useful as a first-line agent in postmenopausal women instead of tamoxifen. Patients with advanced ER/PR-positive breast cancer were divided into two groups with similar demographics. The first group (313 patients) received fulvestrant as a single 250 mg intramuscular dose once a month. The second group (274 patients) was placed on 20 mg tamoxifen daily. Patients were followed for an average of 14.5 months. In patients with hormone receptor-positive tumours, tamoxifen had a non-significant benefit versus fulvestrant in terms of clinical benefit (62.7% versus 57.1%), with the overall conclusion being that fulvestrant had similar efficacy to tamoxifen in this setting.[17]

## Summary

Based on the studies discussed above, it can be concluded that fulvestrant might potentially be used anywhere in the sequence of hormonal therapy. However, the likelihood of a response to fulvestrant is related to the number of prior antihormonal therapies. There is a higher probability of a response after a patient has responded to one therapy compared with after two successive therapies.

The question of what to do following fulvestrant failure is currently under investigation. Patients who initially respond to fulvestrant but subsequently have disease progression are likely to have breast cancer cells that retain their ER/PR positivity. These cancer cells are likely to respond to other endocrine treatment whether fulvestrant is used as a second-line or first-line agent.[18] Finally, because of the time required to reach a steady state with fulvestrant, questions have arisen about response times with fulvestrant and other agents.

Anastrozole takes about 7 days to reach a steady state,[19] compared with fulvestrant which takes up to 6 months.[11] A retrospective study to analyse time to response in the trials that compared fulvestrant, anastrazole and tamoxifen indicated that these were similar for all three drugs.[20] New phase III trials are underway to evaluate the use of a 500 mg loading dose followed by 250 mg on days 14, 28 and monthly thereafter to see if the time to response can be decreased with fulvestrant.[20] Although fulvestrant has demonstrated that it is effective for patients who have failed prior hormonal therapy, new strategies to determine the optimum sequencing of this drug continue. Nevertheless, as our understanding of cellular signalling increases and newer targeted therapies evolve, the possibility of combining growth factor inhibitors with anti-oestrogens such as fulvestrant may offer benefits for disease control in the future.[21,22]

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## JOURNAL OF CLINICAL ONCOLOGY

### SPECIAL ARTICLE

# The 38th David A. Karnofsky Lecture: The Paradoxical Actions of Estrogen in Breast Cancer—Survival or Death?

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### ABSTRACT

During the first David A. Karnofsky Award lecture entitled "Thoughts on Chemical Therapy" in 1970, Sir Alexander Haddow commented about the dramatic regressions observed with estrogen in some breast cancers in postmenopausal women, but regrettably the mechanism was unknown. He was concerned that a cancer-specific target would remain elusive, without tests to predict response to therapy. At that time, I was conducting research for my PhD on an obscure group of estrogen derivatives called nonsteroidal antiestrogens. Antiestrogens had failed to fulfill their promise as postcoital contraceptives and were unlikely to be developed further by the pharmaceutical industry. In 1972, that perspective started to change and ICI 46,474 was subsequently reinvented as the first targeted therapy for breast cancer. The scientific strategy of targeting the estrogen receptor (ER) in the tumor, treating patients with long-term adjuvant therapy, examining active metabolites, and considering chemoprevention all translated through clinical trials to clinical practice during the next 35 years. Hundreds of thousands of women now have enhanced survivorship after their diagnosis of ER-positive breast cancer. However, it was the recognition of selective ER modulation (SERM) that created a new dimension in therapeutics. Nonsteroidal antiestrogens selectively turn on or turn off estrogen target tissues throughout the body. Patient care was immediately affected by the recognition in the laboratory that tamoxifen would potentially increase the growth of endometrial cancer during long-term adjuvant therapy. At that time, a failed breast cancer drug, keoxifene, was found to maintain bone density of rats (estrogenic action) while simultaneously preventing mammary carcinogenesis (antiestrogenic action). Perhaps a SERM used to prevent osteoporosis could simultaneously prevent breast cancer? Keoxifene was renamed raloxifene and became the first SERM for the treatment and prevention of osteoporosis as well as the prevention of breast cancer, but without an increase in endometrial cancer. There the story might have ended had the study of antihormone resistance not revealed a vulnerability of cancer cells that could be exploited in the clinic. The evolution of antihormone resistance over years of therapy reconfigures the survival mechanism of the breast cancer cell, so estrogen no longer is a survival signal but a death signal. Remarkably, remaining tumor tissue is again responsive to continuing antihormone therapy. This new discovery is currently being evaluated in clinical trials but it also solves the mystery mechanism of chemical therapy with estrogen noted by Haddow in the first Karnofsky lecture.

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### INTRODUCTION

By looking back, we can see the way forward. In 1970, Sir Alexander Haddow, FRS presented the first David A. Karnofsky Memorial Lecture entitled "Thoughts on Chemical Therapy."[1] Paul Ehrlich, MD, was the individual who revolutionized therapeutics when he first created a "chemotherapy" (chemical therapy) through rational synthesis, followed by predictive testing in laboratory models, and then clinical trials to demonstrate the cure of syphilis with Salvarsan.[2] He next turned to the treatment of cancer, but after more than a decade, he declared the year before he died in 1915: "I have wasted fifteen years of my life in experimental cancer research."[3] In his Karnofsky lecture, Haddow echoed Ehrlich's sentiment with the statements "the fact that the cancer cell is but a modification of the normal somatic cell holds out little prospect of a chemotherapia specifica in Ehrlich's sense" and "the need exists for some method of prior screening to indicate the optimal choice (of chemotherapy) in particular cases. . . . efforts thus far have been disappointing."[1] Haddow did, nevertheless, mention his results with the first chemical therapy for the treatment of any cancer—high-dose estrogen therapy. Haddow's work in 1944[4] showed that 25% of patients with advanced breast cancer treated with high doses of estrogen had clear responses. In 1944, the steroid estradiol was not available for therapeutics. Instead,

synthetic estrogens called triphenylethylenes (made by Imperial Chemical Industries [ICI], now AstraZeneca) were used because they were cheap, effective, and long acting. Haddow noted "the extraordinary extent of tumor regression observed in perhaps 1% of postmenopausal cases has always been regarded as of *major theoretical importance* and it is a matter of *some disappointment that so much of the underlying mechanisms continue to elude us.*"[1] It should be stressed that Haddow's studies were a paradox, as a link between ovarian estrogen and breast cancer growth had already been established.[5-7] What was the mysterious anticancer mechanism of high doses of synthetic estrogens?

On the other side of the Atlantic in England, armed with a Medical Research Council Scholarship, I was struggling with a PhD thesis (1969 to 1972) entitled "Structure activity relationships of some substituted triphenylethylenes" at the University of Leeds. These estrogenic compounds had evolved into contraceptives or morning after pills, but had failed because they did the exact opposite in women— they induced ovulation.[8] No one was recommending a career studying triphenylethylenes in 1972; in fact, only after repeated failures did the Leeds University Medical School secure an examiner for my thesis. He was Arthur Walpole, PhD, who many years before had been interested in cancer therapy[9] but, in 1972, was Head of the Fertility Control program at ICI. He had discovered a triphenylethylene derivative, ICI 46,474, a contraceptive in rats which failed in that indication in women. ICI 46,474 was a drug looking for an application. as an antiestrogen,[10] so it could possibly be useful as palliative therapy for advanced breast cancer. However, no laboratory studies then supported this indication.

From the age of 16, I was completely enthralled with organic chemistry, but I wanted to apply chemical therapy to treat cancer. This was a very unfashionable career choice in the 1970s (Table 1) and there were no career opportunities for me at that time. Only a 2-year appointment at the Worcester Foundation for Experimental Biology in Massachusetts to work with Mike Harper (the other patent holder of ICI 46, 474) would change everything. Harper had left the Foundation when I arrived in September 1972, and I was told that I could do anything I wanted for 2 years. I chose to call Arthur Walpole about converting ICI 46,474 into a breast cancer drug but targeted to estrogen receptor (ER)–positive disease in patients.[11] What I did not know at the time was that the administration at ICI had terminated the clinical development program but Walpole had threatened to resign unless the orphan project went forward.[11,12] My call, and our friendship, secured funding to conduct the first systematic laboratory study of the potential applications of ICI 46,474 as a targeted anticancer

agent.[12] No studies in this area other than antifertility studies were conducted by ICI staff. The subsequent continuing investment by ICI Pharmaceuticals Division in my laboratory at the University of Leeds (Pharmacology Department, 1974 to 1979) would shape the clinical application of tamoxifen as a long-term adjuvant therapy[13,14] targeted to the ER[15] and as the first agent approved to reduce the incidence of any cancer in high risk pre- and postmenopausal women.[16-19]

## TRANSITION TO TAMOXIFEN

A number of laboratory principles were defined in the 1970s during the evaluation of tamoxifen's antitumor pharmacology. These principles would ultimately have implications for the successful application of tamoxifen as an adjuvant therapy and as a chemopreventive agent in women at high risk for breast cancer. At that time, the principles as a whole were not embraced by the clinical community primarily because nearly all hopes were pinned on combination cytotoxic chemotherapy to cure both metastatic breast cancer and node-positive breast cancer.[20] A palliative "hormone" (as tamoxifen was then classified) was unlikely to provide benefit. The key to success was the application of the antiestrogen to patients with a potentially responsive tumor (ER positive), with micrometastatic disease (stage I/II) but for the appropriate duration of adjuvant treatment.

In the 1960s, there was sufficient evidence to conclude that some breast cancers grew in response to estrogenic hormones.[21] The discovery of the ER[22] and the development of the ER assay[21] to predict which patients would not respond to endocrine ablative surgery became an important practical advance. The idea was simple. Patients whose tumors had no ERs would not respond to estrogen withdrawal because estrogen was not required for tumor growth. An unnecessary ablative operation (oophorectomy, adrenalectomy, or hypophysectomy) would be avoided.[23] At that time, the clinical application of nonsteroidal antiestrogen (triphenylethylene derivatives) as breast cancer therapies were disappointing with numerous toxic adverse effects,[11] except for ICI 46,474.[24,25]

Lois Trench was the first drug monitor for ICI 46,474 in the United States, and in general, she played a pivotal role in the development of tamoxifen. Specifically, she arranged for ER-positive breast tumors to be dispatched to my laboratory at the Worcester Foundation. I also went to Elwood Jensen's laboratory at the Ben May Laboratory for Cancer Research (University of Chicago) to learn sucrose density gradient analysis to measure ERs in breast tumors and to learn how to create hormone-dependent tumors in rats by the oral administration of the mammary carcinogen dimethylbenzanthracene (DMBA).[26] Armed with these techniques, I returned to the Worcester Foundation and, with resources from ICI Americas, my laboratory demonstrated that tamoxifen blocked estrogen binding to the human tumor ER[15] and that two sustained release injections of tamoxifen would almost completely prevent rat mammary carcinogenesis.[16,17] Lois Trench arranged for me to introduce tamoxifen first to the Eastern Cooperative Oncology Group in 1974,[27,28] and I was subsequently asked to introduce the pharmacology of tamoxifen to the National Surgical Adjuvant Breast and Bowel Project in 1976.[29] This started an association with both organizations that developed the idea of long-term adjuvant tamoxifen therapy[30-32] and more recently, breast cancer risk reduction with the selective ER modulators (SERMs) tamoxifen and raloxifene.[33]

**Table 1.** Clinical Situation in 1972 for the Treatment of Breast Cancer

| Treatment | Fact |
| --- | --- |
| Cytotoxic chemotherapy | An appropriate strategy to kill cancer cells |
| | Kills all rapidly replicating cells with no targeting to cancer |
| Estrogen receptor | Not yet a target for antiestrogenic drugs or even an established predictive test for endocrine ablation for breast cancer treatment |
| | Antiestrogens are failed contraceptives |
| Chemotherapy | The way to cure cancer |

## CLINICAL OUTCOMES WITH TAMOXIFEN

The idea that tamoxifen should be applied as a long-term adjuvant therapy for patients with ER-positive primary breast cancer was first publicly presented in the United Kingdom at Cambridge University in September, 1977[34] and subsequently at the second Adjuvant Therapy of Cancer Meeting in Tucson, AZ, in 1979.[35] The specific conclusion, based on the DMBA model system, was that long-term tamoxifen was the most effective suppressant of occult mammary tumor growth and short-term therapy was unlikely to be effective in clinical trial. At that time, in the mid-1970s, there were sincere concerns that only short-term therapy with tamoxifen should be tested because the drug was effective only in 30% of unselected patients and the average duration of the response was only about 1 year. Longer therapy was "guaranteed" to encourage the rapid development of drug resistance in the occult micrometastases. Michael Baum, who led the NATO group, (Nolvadex Adjuvant Trial Organization, but called NATO to enhance the likelihood that US clinicians would read the papers in the erroneous belief that it was a US clinical trials organization) was the first to report that 2 years of tamoxifen enhanced survival of unselected patients with breast cancer.[36] However, it was the report from the Scottish Trials Office[37] (by coincidence, on my birthday, July 25, 1987) that definitively showed a remarkable survival advantage for unselected women who received 5 years of adjuvant tamoxifen compared with a control group who only received tamoxifen on disease recurrence. Longer was better than shorter therapy, as none of the 1-year adjuvant trials showed a survival benefit; only the overview analysis of randomized clinical trials showed a clear pattern of success for the laboratory concept, especially in premenopausal women with ER-positive breast cancer.[14,38]

Interest in developing a strategy to address the chemoprevention of breast cancer grew and evolved during the early years of the 1980s.[39]

However, based on the laboratory data with the DMBA-induced rat mammary carcinoma model[16,17] and the subsequent finding that tamoxifen inhibited the development of contralateral primary breast cancer,[40] Trevor Powles, at the Royal Marsden Hospital in England, initiated the first pilot study in high-risk women[41] to ascertain volunteer compliance and to eventually address issues of cardiovascular and gynecological safety and the effects of tamoxifen on bone density.[42-44] In contrast, studies conducted at the Wisconsin Comprehensive Cancer Center followed the translational research path from the laboratory to the clinic (see SERM: Laboratory Observations to Clinical Practice). Overall, the published safety data (with the exception of tamoxifen-induced rat liver cancer[45-48]) translated from the laboratory[10,49-51] to patients[41,48,52-54] and provided an appropriate basis to advance chemoprevention trials. Although the Fisher et al study[18,19] was definitive and the most comprehensive, several smaller studies supported the general conclusions that tamoxifen reduced the risk of breast cancer, not only during treatment[55] but for perhaps a decade thereafter when drug-related adverse effects are minimal.[19,56,57]

What has been learned through the experience of adjuvant tamoxifen treatment is that compliance is essential to receive the full benefit of long-term therapy, and that longer therapy is better than shorter therapy.[14,38] Early studies demonstrated that metabolic tolerance to long-term adjuvant tamoxifen treatment does not occur even after a decade of treatment.[30,58] In other words, tamoxifen does not get metabolized to estrogen-like metabolites or become rapidly excreted. However, there are wide interpatient variations in circulating levels of both tamoxifen and metabolites, which this has been a mystery until recently. Hot flashes, or other menopausal symptoms, are the main reason for stopping therapy prematurely, but as it turns out, menopausal symptoms are associated with a good prognosis and with an improved control of disease recurrence.[59,60]
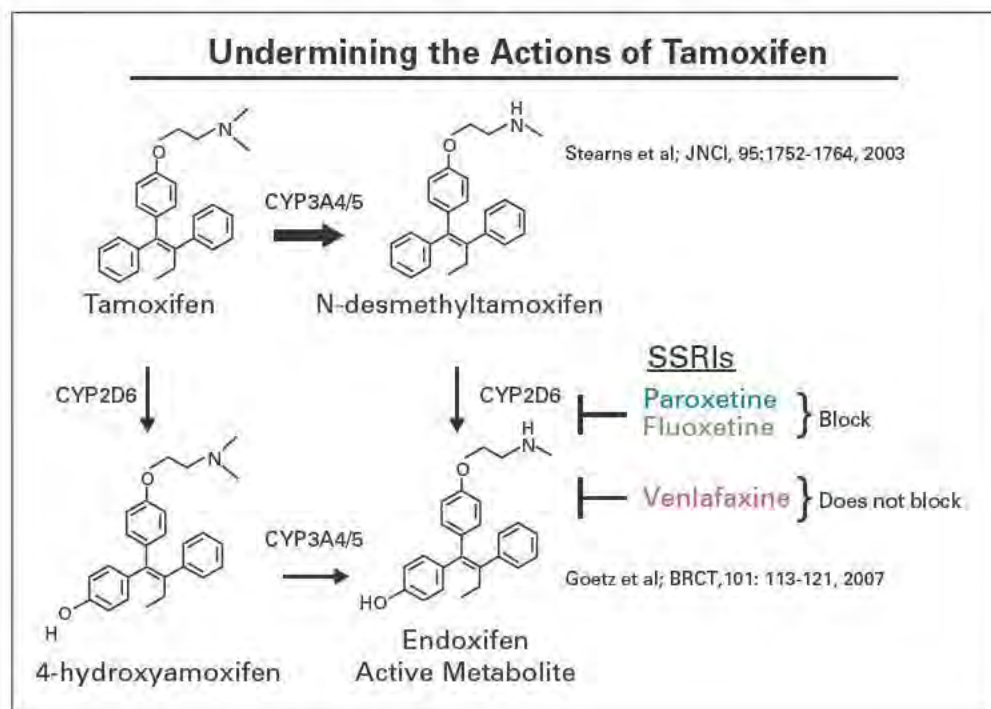


**Fig 1.** The metabolism of tamoxifen and the potential of selective serotonin reuptake inhibitors (SSRIs) to block metabolism of tamoxifen to endoxifen. Venlafaxine has a low affinity for the *CYP2D6* gene product so this is the agent of choice to block hot flashes.

The metabolites of tamoxifen are antiestrogenic (Fig 1) and the conversation of tamoxifen to 4-hydroxytamoxifen is an advantage—but not a requirement—for antiestrogenic activity.[61,62] 4-hydroxytamoxifen continues to be an important laboratory tool for the laboratory study of antiestrogen action[63,64] and has been used to study the crystal structure of the ER with estrogens and antiestrogens.[65] However, a related metabolite endoxifen or 4-hydroxy-N-desmethyl tamoxifen[66] is the major antiestrogenic metabolite of tamoxifen in patients and is produced by the enzyme CYP2D6 (Fig 1).[67] Variants of the enzyme can either increase or decrease tamoxifen metabolism in patients producing more or less endoxifen. It is believed that elevated endoxifen can cause hot flashes which may suggest that the application of a selective serotonin reuptake inhibitor (SSRI) to alleviate these symptoms would be a reasonable course of action to maintain patient compliance. However, certain SSRIs, such as fluoxetine and paroxetine, block CYP2D6 and are contraindicated for patients taking adjuvant tamoxifen (Fig 1).[68-70] Venlafaxine is the SSRI of choice because it has a low affinity for CYP2D6. The general principle is to ensure appropriately high levels of endoxifen are produced to provide the best chance for therapeutic success with tamoxifen (Fig 1).

## SERM: LABORATORY OBSERVATIONS TO CLINICAL PRACTICE

The received wisdom in the 1980s was that estrogen could prevent both osteoporosis and coronary heart disease (the latter was subsequently proven to be incorrect in the Women's Health Initiative nearly two decades later).[71] The proposed clinical evaluation of tamoxifen, a so-called antiestrogen, as a chemopreventive in healthy pre- and postmenopausal women, raised the concern that an antiestrogen would prevent the development of breast cancer, but increase the risk of crushing osteoporosis and death from coronary heart disease. In my laboratory at the Wisconsin Comprehensive Cancer Center (Madison, WI), we initiated a program to evaluate the pharmacology of tamoxifen so we could predict the extent of toxic adverse effects in subsequent clinical trials. At that time, we were positioning the overall program at Wisconsin to conduct a chemoprevention study.

We discovered that tamoxifen exhibited target site-specific actions as an estrogen in the mouse uterus[72] and human endometrial cancer,[50] as an antiestrogen in rat mammary carcinogenesis[13,17,73] and in human breast cancer cells,[72] but was an estrogen-like drug able to preserve bone density in ovariectomized rats.[49] Our findings that the target-specific action of tamoxifen-induced endometrial cancer growth[50] had immediate clinical consequences that were to improve health care.[74,75] The public discussions that followed caused clinical trials organizations to evaluate their emerging data. An elevated incidence of endometrial cancer in postmenopausal patients was noted in those women who received tamoxifen.[51,76] Initially, the description of this adverse effect caused unprecedented concern that there would be a high incidence of poor-grade endometrial cancer,[77] but the results of Fisher et al's chemoprevention study[18] clearly demonstrated that there was no elevation in endometrial cancer in premenopausal women, but a four- to five-fold increase in endometrial cancer with good grade (early detection) in postmenopausal women. The involvement of gynecologists in the treatment plan for breast cancer provided the necessary safeguards for patients. Overall, it is now established that the benefits of long-term adjuvant tamoxifen treatment far outweigh the

risks of endometrial cancer,[14,38] but it was clear even in 1989 that an alternative approach to chemoprevention was necessary.[78,79] The idea was simple: "We have obtained valuable clinical information about this *group* of drugs that can be applied in other disease states. Research does not travel straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high-risk group to prevent breast cancer."[79]

This strategic prediction was not made in isolation. We had already completed laboratory studies with a chemical cousin of tamoxifen, called keoxifene, to show it prevented rat mammary carcinogenesis[73] and almost completely blocked tamoxifen-stimulated endometrial cancer growth[80] but prevented bone loss in ovariectomized rats.[49,81] However, at that time in 1990, nobody cared.

## KEOXIFENE RESURRECTED AS RALOXIFENE

The compound known as LY156758 or keoxifene[82] started life as an antiestrogen and all initial efforts in testing were focused on an application as a breast cancer drug. It was to be a competitor for tamoxifen. However, keoxifene failed in that application[83] because the drug group has poor bioavailability[84] and crossresistance with tamoxifen.[85] As with tamoxifen, keoxifene was a drug looking for an application. Scientists at Eli Lilly eventually confirmed[86] the earlier results that keoxifene preserved bone density[49] and like tamoxifen[10] also lowered circulating cholesterol (tamoxifen already had a patent as a hypocholesteremic agent[11]).

The trial Multiple Outcomes of Raloxifene Evaluation (MORE) addressed the hypothesis that raloxifene could reduce the incidence of fractures in high-risk osteoporotic postmenopausal women. The results showed raloxifene did reduce spinal fractures by approximately 50% during the 3-year treatment period.[87] Raloxifene was the first SERM approved to treat and prevent women at risk for osteoporosis. The second preplanned evaluation was breast and endometrial safety. I was the chair of the Oncology Advisory Committee established to monitor breast cancer incidence. We found a significant 70% decrease after 3 years of raloxifene[88] in the incidence of breast cancers and after 4 years[89] of raloxifene treatment for osteoporosis. A subsequent evaluation of a placebo-controlled trial called Raloxifene Use for the Heart (RUTH), designed to evaluate the cardio protective actions of the SERM,[90] also noted a significant decrease in invasive breast cancer incidence and more importantly, both MORE[88] and RUTH[90] showed no elevation in endometrial cancers. However, the RUTH trial showed no improvement or benefit for patients at risk for dying from cardiac disease if they took raloxifene.[90]

As a public health intervention, the original proposal[78,79] that a SERM used to prevent osteoporosis in women at risk for osteoporosis could simultaneously reduce the incidence of breast cancer appears to be valid. With the current shift in the prescribing of hormone replacement therapy in the wake of the Women's Health Initiative[71] in the

United States and the Million Women's Study[91] in the United Kingdom, a decrease in the incidence of ER-positive breast cancer has been noted by Ravdin.[92] With the availability of raloxifene as long-term therapy to treat and prevent osteoporosis, it is clear that there will potentially be a reduction in breast cancer incidence in the general population. This anticipated decrease in breast cancer incidence with long-term raloxifene use is evidenced by the data published by Martino et al.[93] These data were recently used to estimate decreases in breast cancer incidence in large populations of women not identified as at risk for breast cancer.[94]

The good safety and efficacy profile for raloxifene made it the agent of choice to compare head-to-head against tamoxifen in the Study of Tamoxifen and Raloxifene (STAR) to reduce breast cancer incidence in postmenopausal women deemed at high risk. Norman Wolmark invited me to be the scientific chair on the STAR trial advisory board just in case there were any toxicological or pharmacologic surprises. None occurred. Overall, the results[33] were another important step forward in chemoprevention; tamoxifen and raloxifene reduced the incidence of breast cancer equally, but the safety profile of raloxifene is superior. Based on the clinical trials,[19,33,55,93,95] it is now possible to summarize progress in chemoprevention (Table 2[19,33,39,88,96]), because agents can now be applied selectively to patient populations. However, each agent has been reinvented and then transitioned from the laboratory through clinical trials to an advance in health care, a process that extended over 30 years. It is perhaps important to state that the prudent use of tamoxifen or raloxifene to reduce the risk of breast cancer in the appropriate groups of high risk women is an important advance in therapeutics. Regrettably, there is reluctance to use these approved agents within the high-risk population, but often this is because of misinformation about the risks as physicians are now in a position to pick the right agent for the right patient.

## DRUG RESISTANCE TO SERMS

The acceptance of the concept of long-term antihormone therapy to target, treat, and prevent breast cancer[20] raised the specter of drug resistance to SERMs. Twenty years ago, my team took a long-term view by creating a whole range of breast and endometrial cancer models resistant to tamoxifen and raloxifene.[97-101] Our goal was to anticipate the clinical development of drug resistance and to understand mechanisms so that second-line therapies could be deployed rationally. The models were developed naturally by first establishing estrogen stimulated tumor growth in athymic mice followed by long-term SERM treatment to identify SERM-resistant tumors. All our models were retransplanted into subsequent generations of mice so that the impact of long-term SERM therapy could be evaluated in

hormone-responsive breast and endometrial cancer. What is unique about SERM resistance is that both breast and endometrial tumors grow in response to either SERMs or estrogen. No estrogen (mimicking aromatase inhibitor treatment) or the use of a pure antiestrogen (ICI 164,384[102] or fulvestrant[103,104]) prevent SERM resistant tumor growth. This is why aromatase inhibitors or fulvestrant are effective second-line therapies after tamoxifen failure.[105,106]

However, the early models of SERM resistance did not reflect the majority of clinical experience. The natural laboratory models developed during a year of therapy[97,107] and therefore reflected drug resistance in patients with metastatic breast cancer who are only treated successfully for 1 year. In other laboratories, ER-positive models were developed that were engineered by stable transfection of the HER2/neu gene.[108,109] These tumors are resistant to tamoxifen but reflect a small subset of clinical disease, including ER/HER2/neu–positive breast cancer. We took the strategic decision to determine what would occur if breast tumors were retransplanted into successive generations of tamoxifen stimulated mice for 5 years or more (ie, to replicate the actual clinical conditions employed during long-term adjuvant therapy). Remarkably, drug resistance evolves (Fig 2[99,110]) and the survival signaling pathway in tamoxifen resistant tumors becomes reorganized so that instead of estrogen being a survival signal, physiologic estrogen now inhibits tumor growth. This discovery[99,111] provided an invaluable insight into the evolution of drug resistance to SERMs and prompted the reclassification of the process through phase I (SERM/estrogen stimulated growth) and phase II (SERM stimulated growth estrogen inhibited growth). This new knowledge now provides an opportunity to treat patients with low-dose estrogen after exhaustive antihormone therapy.

## NEW BIOLOGY OF ESTROGEN ACTION: CLINICAL TRANSLATION

The apoptotic action of physiological estrogen to cause dramatic tumor regression of long-term tamoxifen-resistant ER-positive breast cancers grown in athymic mice[99,111] was subsequently extended to long-term raloxifene resistance[112] and ER-positive breast cancer cells maintained in an estrogen-deprived environment for prolonged periods.[110,113-116] Most importantly, the apoptotic results observed with estrogen-deprived cells were noted both in vitro and in vivo by inoculation into athymic mice.[110]

Mechanistic studies, using our unique laboratory models, demonstrate that the antihormone resistant cells have reconfigured the ER signal transduction pathway so despite the fact that the ER still regulates the appropriate estrogen-regulated genes (including $pS_2$ and $myc$)[117] there is a profound effect of estrogen to activate the fas (death)

| Table 2. Practice of Prevention 2008[19,33,39,88,94,96] | | |
| --- | --- | --- |
| Drug | Group/Reason | Advantage |
| Tamoxifen | High-risk postmenopausal women | No increase in blood clots or endometrial cancer |
| Raloxifene | High-risk premenopausal women | No increase in endometrial cancer |
| | Treatment and prevention of osteoporosis | Reduction in the risk of breast cancer and no increase in endometrial cancer |
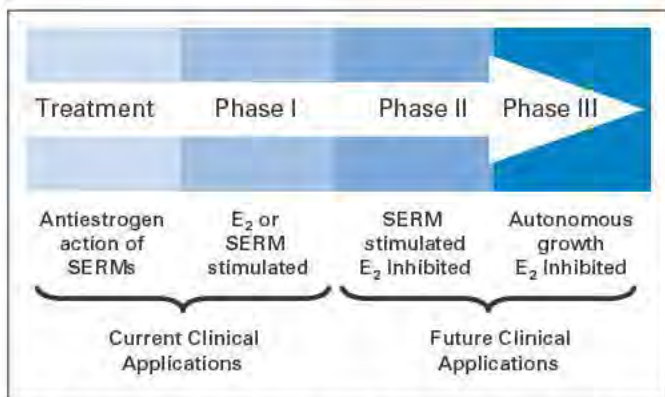
Fig 2. Evolution of drug resistance to selective estrogen receptor modulations (SERMs). Acquired resistance occurs during long-term treatment with a SERM and is evidenced by SERM-stimulated breast tumor growth. Tumors also continue to exploit estrogen for growth when the SERM is stopped, so a dual signal transduction process develops. The aromatase inhibitors prevent tumor growth in SERM-resistant disease and fulvestrant that destroys the estrogen receptor (ER) is also effective. This phase of drug resistance is referred to as phase I resistance. Continued exposure to a SERM results in continued SERM-stimulated growth, but eventually autonomous growth occurs that is unresponsive to fulvestrant or aromatase inhibitors. The event that distinguishes phase I from phase II acquired resistance is a remarkable switching mechanism that now causes apoptosis, rather than growth, with physiologic levels of estrogen. These distinct phases of laboratory-drug resistance[99,110] have their clinical parallels and this new knowledge is being integrated into the treatment plan.



Fig 3. Clinical protocol to investigate the efficacy of estradiol induced apoptosis in long-term endocrine refractory breast cancer. An anticipated treatment plan for third-line endocrine therapy. Patients must have responded and experience treatment failure with two successive antihormone therapies to be eligible for a course of low-dose estradiol therapy for 3 months. The anticipated response rate is 30%[121] and responding patients will be treated with anastrozole until relapse. Validation of the treatment plan will establish a platform to enhance response rates with apoptotic estrogen by integrating known inhibitors of tumor survival pathways into the 3-month debulking "estrogen purge". The overall goal is to increase response rates and maintain patients for longer on antihormone strategies before chemotherapy is required.

receptor system[115,118] or to alternatively have a direct effect on mitochondrial function via the bcl2 system.[111,119] Thus, an understanding of the paradoxical actions of estrogen has emerged that depend on the state of estrogen deprivation of the breast cancer cell. In an estrogen rich environment, the estradiol-ER complex is a survival system promoting tumor growth. In contrast, in an estrogen-deprived environment (treatment with tamoxifen or an aromatase inhibitor) estrogen action is replaced by internal survival signaling based on the selection of cells with enhanced growth factor receptors. The growth factor receptors[120] initiate cascades that phosphorylate either unoccupied ER or ER liganded by SERMs. This model would also explain the earlier observations why high-dose estrogen therapy was only effective as a treatment for breast cancer in women many years after the menopause.[1] Natural estrogen deprivation had occurred. The process is accelerated and enhanced, however, in patients treated long-term with SERMs or aromatase inhibitors so that only low doses of estrogen are necessary to cause experimental tumors to regress. The question now becomes, can this new laboratory knowledge be translated to patient care?

Several clinical trial groups are currently addressing this issue. In our own case, we are recruiting patients with metastatic breast cancer who have succeeded and experienced treatment failure with at least two successive endocrine therapies (Fig 3) and we are determining the efficacy of a 12-week purge of high-dose estradiol (30 mg daily) therapy. The goal is to confirm and extend the previously study published by Lonning and colleagues[121] and then to determine the minimum dose of estradiol necessary to induce the anticipated 30% response rate.[121] Based on our previous laboratory studies,[99] we propose to retreat responding patients with anastrozole to determine efficacy.

Overall, our clinical program is part of a multi-institutional Center of Excellence grant BCO50277 entitled "A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced

Apoptosis" that will map the survival and death pathways of our models and integrate clinical material to determine the validity of the laboratory-derived molecular mechanisms and, ultimately, to address the issue of why the majority of tumors do not respond to estrogen alone. Knowledge of the new apoptotic biology of estrogen could be enhanced in the future in much the same way as the modest responses initially observed were enhanced to benefit patients with tamoxifen and raloxifene. The philosophy is to deploy the right treatment at the right time and for the right patient.

## PROGRESS IN TREATING DISEASE?

In closing, it is perhaps pertinent to re-examine Haddow's comments delivered during the first David A. Karnofsky lecture in 1970. He saw little evidence that specific chemical therapies could be developed and there was really no predictive test to identify tumors that could respond to a chemical therapy. The idea of a targeted drug was to be advanced soon thereafter during the 1970s[20] when the ER assay evolved from being a predictive test for endocrine ablation to become the target for a failed contraceptive to be reinvented as tamoxifen and to be used for long durations in the treatment and prevention of breast cancer.[11] However, translational research does not travel in straight lines: one needs luck so the unanticipated can be integrated into the treatment plan and perhaps, if one is lucky, new innovations in therapy can be developed.

SERM was unanticipated and much luck led to progress in treatment. Issues over the increased risk of endometrial cancer caused by tamoxifen treatment coupled with the recognition that the drug group called the nonsteroidal antiestrogens[122] could enhance bone density in animals[49,123] and man[54] opened the door for the development of raloxifene[81] as the first SERM for the treatment and prevention of osteoporosis as well as the reduction of risk for breast cancer,[33,88] but

with no increase in endometrial cancer risk. Chemoprevention has now extended from an idea[16,17,124] to a clinical reality (Table 2).

The enormous impact that tamoxifen has had on the treatment of breast cancer for 25 years (1978 to 2003) naturally encouraged efforts to improve treatment responses and reduce the adverse effects noted with tamoxifen.[125] This goal has been achieved with the introduction of a range of aromatase inhibitors for the treatment of breast cancer in postmenopausal women.[125,126] The principles of treatment remain the same: targeting the ER and then employing long-term therapy now for perhaps up to 15 years to exploit the trend observed in MA-17 (tamoxifen followed by an aromatase inhibitor).[127] Tamoxifen surprisingly did not go away, but remains the treatment of choice for premenopausal women with breast cancer, the appropriate agent for risk reduction in premenopausal women, a major drug of interest for the study of pharmacogenomics, and the major life-saving antihormone in countries throughout the world that do not have the sophisticated and wealthy health care system we have in the United States. Furthermore, the laboratory principle from the 1970s that "longer is better" for adjuvant therapy[13,128] continues to be evaluated in the Adjuvant Tamoxifen Long Against Short (ATLAS) trial that compares 10 years of tamoxifen with 5 years of tamoxifen. If 10 years of tamoxifen treatment is superior to 5 years, then the public health impact will be profound as this cheap and easily accessible drug can continue to provide benefit in lives saved. The current approaches and advances in the antihormone therapy of breast cancer are summarized in Figure 4.

Finally, the paradox of estrogen action in dictating the survival or death of breast cancer cells has become transparent, closing a circle of knowledge left hanging in the wake of Haddow's Karnofsky presentation in 1970.[1] The dramatic results he observed with high-dose estrogen therapy in a small fraction of women[1] was a powerful testament to the potential of chemical therapy. Unfortunately, there was no knowledge about the mechanisms to further exploit the concept. Fashions in therapy began to move toward blocking estrogen action and shifted from the more toxic high doses of estrogen to the less toxic but equally efficacious tamoxifen.[129] Now we find ourselves returning to the beginning of "chemical therapy" because unusual and unanticipated laboratory observations were placed on the web of knowledge. This knowledge has remained dormant until it could now be called to the center of the web when the fashion in research again changes. The discovery of apoptosis as a natural process to destroy aberrant cells[130] would probably have never be linked in the same sentence with "hormone" therapy. However, it is now clear that antihormone drug resistance can reprogram some hormone responsive cancer cells to be supersensitive to the apoptotic actions of physiological estrogen.[99,111] These tantalizing laboratory observations now provide another opportunity for chemical therapy to aid patients. The knowledge is already finding its way into clinical trials, so that in the future it may be possible that the antihormone resistant disease from select patients can be destroyed by an "estrogen purge" and then patients could again be maintained for a longer period on an antihormone therapy.



**Treatment Practice 2008**
Long-Term Estrogen-Deprivation Treatment

A
Tamoxifen (5 years)
Aromatase Inhibitor (5 years)

ATAC, *The Lancet*, 359:2131-40, 2002
Howell et al, *The Lancet*, 365:60-2, 2005
Thurlimann et al, *N Engl J Med*, 353:2747-57, 2005
Coates et al, J Clin Oncol, 25:496-492, 2007

B
Tamoxifen Aromatase Inhibitor
(2-3 years)    (3 years)

Coombes et al, *N Engl J Med*, 350:1081-92, 2004
Boccardo et al, *J Clin Oncol*, 23:5138-47, 2005

C
Tamoxifen
(5 years)

Goss et al, *N Engl J Med*, 349:1793-802, 2003
Goss et al, J Natl Cancer Inst, 97:1262-71, 2005

Tamoxifen    Aromatase Inhibitor    Different Aromatase Inhibitor
(5 years)    (5 years)    (Additional 5 years)

D
Tamoxifen
(5 years)

Atlas Trial

Tamoxifen
(10 years)

Fig 4. Adjuvant antihormone strategies for the treatment of estrogen receptor–positive breast cancer.[126,127] ATLAS, Adjuvant Tamoxifen Long Against Short.

We have perhaps researched the zenith of our abilities to manipulate the ER with our current armamentarium. So, is this then the end of our story? Certainly not. There is much still to be accomplished. The SERM concept has now been extended to include all members of the steroid receptor superfamily[20,131] so that in the future diseases may be selectively treated that until now had been thought to be untreatable. New specific medicines are now being developed to achieve this goal.[131,132] But, where could the estrogen-induced apoptosis story take us? It may be that the modest results observed in select sensitive patients with ER-positive metastatic breast cancer could be amplified by the prudent use of selective survival inhibitors. If the cancer cell is prevented from surviving, then perhaps the mild estrogen apoptotic trigger will kill more tumor cells. Indeed, if we can work out how the ER complex naturally seeks out its intracellular trigger, then perhaps that trigger could be the next target for chemical therapy for a range of cancers beyond breast cancer.

### AUTHOR'S DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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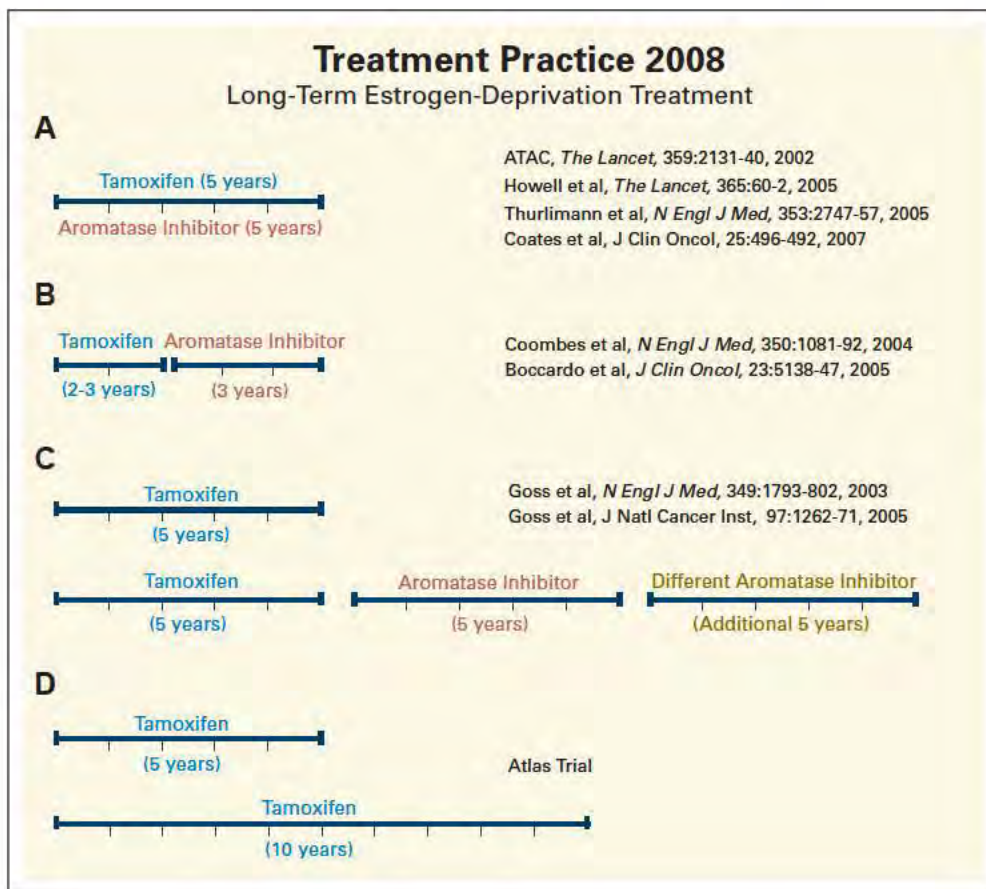**3.** Schrek R: Fashions in cancer research, in The Year Book of Pathology and Clinical Pathology. Chicago, IL, The Year Book Publishers, 1959, pp. 26-39

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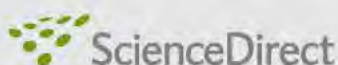
Review

# Tamoxifen: Catalyst for the change to targeted therapy

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ARTICLE INFO

ABSTRACT

In the early 1970s, a failed post coital contraceptive, ICI 46,474, was reinvented as tamoxifen, the first targeted therapy for breast cancer. A cluster of papers published in the European Journal of Cancer described the idea of targeting tamoxifen to patients with oestrogen recep tor positive tumours, and proposed the strategic value of using long term tamoxifen therapy in an adjuvant setting with a consideration of the antitumour properties of the hydroxylated metabolites of tamoxifen. At the time, these laboratory results were slow to be embraced by the clinical community. Today, it is estimated that hundreds of thousands of breast cancer patients are alive today because of targeted long term adjuvant tamoxifen therapy. Addi tionally, the first laboratory studies for the use of tamoxifen as a chemopreventive were pub lished. Eventually, the worth of tamoxifen was tested as a chemopreventive and the drug is now known to have an excellent risk benefit ratio in high risk pre menopausal women. Over all, the rigorous investigation of the pharmacology of tamoxifen facilitated tamoxifen's ubiq uitous use for the targeted treatment of breast cancer, chemoprevention and pioneered the exploration of selective oestrogen receptor modulators (SERMs). This new concept subse quently heralded the development of raloxifene, a failed breast cancer drug, for the preven tion of osteoporosis and breast cancer without the troublesome side effect of endometrial cancer noted in post menopausal women who take tamoxifen. Currently, the pharmaceuti cal industry is exploiting the SERM concept for all members of the nuclear receptor super family so that medicines can now be developed for diseases once thought impossible.

## 1. Introduction

*A new dynasty gives dominion over the ruling dynasty through per severance and not by sudden action* (Ibn Khaldun 14th Century Arab Historian)   and so it is with changes in the approach to cancer therapy. This article will focus specifically on a clus ter of scientific papers[1–3] published in the European Journal of Cancer that presaged the dramatic changes that have oc curred in the past 35 years in our approach to cancer therapy. To set the scene, it is first appropriate to describe the research and treatment philosophy for breast cancer before tamoxifen.

In the 1960s, the use of combination cytotoxic chemother apy for the treatment of breast cancer had moved to centre stage in the wake of an abstract presented at the American Association for Cancer Research.[4] The cytotoxic 'cocktail' pre sented by Cooper, containing cyclophosphamide, methotrex ate, 5 fluorouracil, vincristine and prednisone (CMFVP), produced a dramatic response rate of >80% in patients with advanced breast cancer. In the 1960s, there was every reason to believe that cancer would be curable if (1) the right drug combination could be found; (2) the patient could be kept alive through the aggressive high dose regimens; and (3) pa

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tients could be treated with a low tumour burden. Cytotoxic chemotherapy became king and a new dynasty was established with the initiation of a lexicon of drug combinations and schedules and ultimately, bone marrow transplantation. The introduction of adjuvant therapy, as it turned out, would be essential for the successes we see today when the move occurred from cytotoxic chemotherapy to tamoxifen treat ment. The initial hypothesis for the use of cytotoxic chemo therapy was reasonable and logical; adjuvant chemotherapy would destroy undetected micrometastases harboured around the patient's body after surgical removal of the pri mary tumour. The perfect result would be enhanced cures for women with breast cancer but the biology of breast cancer conspired to defeat the best attempts of oncologists to deploy non specific cytotoxic chemotherapy effectively. The hypoth esis was flawed.

It is the responsibility of each new generation to challenge the fashions in medicine created by the ruling dynasty. Pro gress by defying the dynasty can be profound and today we witness the results of an unlikely revolution in thinking that had its roots in the 1970s. Around the world, death rates from breast cancer are declining and patients are living longer, recurrence free lives with less morbidity. Tamoxifen is an integral reason for current progress, but this was unantici pated in the 1970s. Thirty five years ago it would have been unthinkable to suggest that 'hormone therapy' would en hance survivorship and that breast cancer risk reduction would now be a clinical reality.

Our knowledge of human oncogenes, an unknown idea in 1972 (C src the first oncogene was described in 1976) now pro vides invaluable clues to exploit, selectively, the metabolic vulnerabilities in cancer. This knowledge is creating justifi able optimism by targeting the disease specifically with new agents. The current generation has witnessed the clinical (and economic!) success of agents like trastuzumab that tar gets gene amplified HER2 neu[5] in select breast cancers to pro duce disease control[6–8] not previously thought possible. However, the new era of individualised targeted medicines that promises 'to kill or prevent the cancer but not harm the patient' did not start with biotechnology.

The origins of targeted therapy started in the 1970s by challenging cytotoxic chemotherapy with an alternative ap proach to treatment which was achieved by adapting the pharmacological principles of drug receptor theory to cancer care. At that time, cancer research was considered to be a hopeless career choice, but a series of events put the right people in the right place at the right time to recognise a unique opportunity to advance cancer therapeutics. No advances occur in isolation; they build on the work of previous generations and in this case, by collegial inter action.

## 2. Tamoxifen (ICI 46,474) before targeting

ICI 46,474, the antioestrogenic *trans* isomer of a substituted triphenylethylene, was discovered in the laboratories of Imperial Chemical Industries (ICI) Ltd. Pharmaceuticals Divi sion (now AstraZeneca). The team, Dora Richardson (Chem ist), Michael J.K. Harper (Reproductive Endocrinologist) and Arthur L. Walpole (Head of Reproduction Research) was tasked with developing a post coital contraceptive during the early 1960s based on the structural clues already pub lished by other pharmaceutical companies. All of the studies conducted at ICI throughout the 1960s were focused on repro duction and the patent issued throughout the world (except the United States where the patent was denied for 20 years because the findings did not demonstrate innovation) stated 'the alkene derivatives of the invention are useful for the modification of the endocrine status in man and animals and they may be useful for the control of hormone dependent tumours or for the management of the sexual cycle and aber rations thereof. They also have useful hypocholesteraemic activity'. Claims that the compounds could be used as a breast cancer treatment had to be removed from the patent applications in America as they were considered to be fantas tic![9] More importantly, there was no evidence to back up the claim.

Walpole was not only interested in reproductive endocri nology but also cancer research and treatment.[10] The scien tists at ICI had found an unusual species specificity with ICI 46,474; the compound was apparently a classical oestrogen in the mouse vagina but an antioestrogen in rat tests.[11,12] The question was what was the pharmacology of ICI 46,474 in humans: an oestrogen or an antioestrogen? Walpole ad vanced clinical testing of ICI 46,474 in both 'the control of hor mone dependent tumours' and 'the regulation of the sexual cycle'. Clinical testing was initiated to evaluate activity to treat breast cancer at the Christie Hospital in Manchester and the Princess Margaret Hospital, Birmingham[13,14] and reproductive cycle studies proceeded elsewhere.[15] In 1972, all conclusions were reviewed by ICI Ltd. Pharmaceuticals Division in Alderley Park, Macclesfield, Cheshire. Unlike the results observed in the rat, ICI 46,474[16] was not a contracep tive in humans. The drug induced ovulation and could poten tially be used as a profertility agent.[15] ICI 46,474 exhibited modest activity as a breast cancer therapy which was equiv alent to historical controls treated with high dose oestrogens or androgens.[13] The advantage of tamoxifen, that was to be critical for future applications, was a low incidence of toxic side effects. However, the decision by senior management was to abandon further development,[9,17] primarily because the financial return for co marketing a breast cancer drug used by a limited number of patients for about a year for the palliation of metastatic breast cancer was too small and there was virtually no market for another agent to induce ovulation in subfertile women. Clomiphene was already the medicine of choice.[18]

Walpole responded by electing to take early retirement if ICI 46,474 did not get marketed. He was at the end of his sci entific career and he truly believed that tamoxifen had prom ise if only further studies could be completed on the 'orphan drug'. But how would this occur? Walpole and I met in Sep tember, 1972, when he was the external examiner of my PhD entitled 'Structure function relationships of some tri phenylethylenes and triphenylethanes' at the University of Leeds. Following this meeting, Walpole provided resources for me to conduct the scientific work that reinvented a failed contraceptive to become the first targeted therapy for the treatment and prevention of breast cancer. We collaborated until his untimely death in 1977.[10]

## 3.    Foundations

In 1969, I was seduced by the idea of crystallising the oestro gen receptor (OER) with an oestrogen and a non steroidal antioestrogen. My supervisor thought it would be a little unin teresting, but at least the project would be straightforward as Leeds had a premier X ray crystallography department called the Astbury Department of Biophysics. The OER protein could be easily extracted from uteri,[19,20] but I quickly found that purification was not a simple task. I switched my PhD topic to study the pharmacology of non steroidal antioestrogens. As it turned out, this was a good career choice as no one has yet succeeded in crystallising the whole liganded OER!

I wanted to develop drugs for cancer, but there were no opportunities to pursue this goal during my PhD. What made life more complicated in 1972 was the fact that the University could not find anyone to be my external examiner; no one cared about the pharmacology of failed contraceptives! Although administrators at the University protested against the choice of someone from industry, Arthur Walpole was eventually appointed as my examiner; a fortunate event that was subsequently to advance the clinical application of tamoxifen by establishing a scientific foundation through an investigation of its antitumour actions in the laboratory.

During the final year of my PhD, I was invited to stay at Leeds as a lecturer in Pharmacology. However, first I was re quired to go to the Worcester Foundation for Experimental Biology (now the Worcester Foundation for Biomedical Re search, part of the University of Massachusetts Medical School) to work with Michael Harper, Walpole's former col league at ICI. When I arrived in September 1972, Harper de clared that he had accepted a job at the World Health Organisation in Geneva and that 'I could do anything I wanted for the next two years'.

Here was the opportunity I wanted. A phone call to Walpole at ICI secured his enthusiastic financial support to re examine ICI 46,474 in the laboratory, but this time the focus would be its mechanism of action as an anticancer agent. I was made a consultant to introduce ICI 46,474 to clinical trials groups in American and Lois Trench, the drug monitor for Stuart Phar maceuticals (ICI Americas in Wilmington, Delaware) coordi nated all administrative details between 1972 and 1974 to get the project off the ground. But how to start?

Elwood V. Jensen, Director of the Ben May Research Labora tory was on the scientific advisory board for the Worcester Foundation in 1972 (Fig. 1). During his visit in late 1972, we spent time going over my thesis and I explained what I wanted to do with ICI 46,474. He generously invited me to Chicago the next year to learn sucrose density gradient analysis in order to study whether tamoxifen blocked oestradiol binding to the human and animal OER. I also learned how to induce mam mary tumours in rats using dimethylbenzanthracene (DMBA) so that the mechanism of antitumour action of tamoxifen could be evaluated under controlled laboratory conditions. The DMBA model was the only model available at the time to study hormones and cancer. The work commenced at the Worcester Foundation in the summer of 1973 and by the end of the year, results were pouring out. Lois Trench secured hu man tumours for sucrose density gradient analysis, but I felt no pressure to publish as no one was really interested. Chemo therapy was king and no one anticipated that another 'hor mone therapy' would be an advance. As a pharmacologist, I was just happy to be contributing to the development of an anticancer drug.



Fig. 1 – V. Craig Jordan and Elwood V. Jensen on the occasion of learning they were going to be the inaugural recipients of the Dorothy P. Landon/AACR Prize (2002) for Translational Research. This is the highest award presented by the AACR and recognised the seminal work for both of these scientists; Elwood Jensen identified OER as the mediator of oestrogen action in its target tissues and some breast tumours, and Craig Jordan's research that reinvented ICI 46,474 from being a failed contraceptive to the first targeted therapy for breast cancer as the drug tamoxifen.

Avoiding writing up my results could not last forever. Dr. Eliahu Caspi, a senior scientist at the Worcester Foundation, was directed to interview me to explore the possibility of me staying at the Worcester Foundation and not returning to Leeds. This was a surprise, but there was an even bigger surprise in store when he glared at me over his desk and an nounced 'that I did not have a CV because I had not any pub lications'. I announced I had not yet solved any problems so what was the point? And he proceeded to give me the best ad vice of my academic career up to that time. 'Tell them the story so far; each paper should take no longer than two weeks to write up and link together a series of studies with a theme'. I have not stopped writing since; which brings me back to the three papers I eventually published in the European Journal of Cancer.[1–3]

## 4.    Transition to targeting Tamoxifen (Jordan VC, Koerner S. Tamoxifen (ICI 46,474) and the human carcinoma 8S oestrogen receptor. Eur J Cancer 1975;11:205–6)

Lars Terenius published two important papers in the Euro pean Journal of Cancer that described the action of nafoxidine for the treatment of DMBA induced rat mammary tumours[21] and the ability of the first non steroidal antioestrogen MER 25[22] to prevent rat mammary carcinogenesis.[23] These studies demonstrated 'proof of principle' for the application of anti oestrogens to treat breast cancer, but neither compound showed any promise in the clinic because of serious toxic side effects.[24,25] In fact, this was the consistent story for all of the antioestrogens, except for tamoxifen.

ICI, 46,474 was examined systematically in my laboratory to explore mechanisms and applications that could be exploited in the clinic. These studies were supported by ICI with unrestricted funds, first at the Worcester Foundation (1972 1974) and subsequently at the University of Leeds as a University Joint/Research Scheme (1974 1979). Most impor tantly, ICI arranged for thousands of rats to be chauffeured from Alderley Park to Leeds so I could complete my work. Those free rats, as it turned out, would be worth their weight in gold with the billions of pounds of profits earned with tamoxifen! Simultaneously, Rob Nicholson, at the Tenovus Institute in Cardiff started to use tamoxifen as a laboratory tool to investigate oestrogen and antioestrogen action in the DMBA induced rat mammary tumour model. Again, these studies were published in the European Journal of Cancer.[26–28]

The studies I conducted in the laboratory initially focused on the ER as a therapeutic target. The questions that were ad dressed were 'can tamoxifen block oestrogen binding?' and 'is tamoxifen the active agent?' ICI 46,474 has a very low binding affinity for the ER and we used sucrose density gradient anal ysis to provide the first consistent evidence that tamoxifen blocks the binding of oestradiol to the human breast and endometrial cancer 8S oestrogen receptor.[1] We focused spe cifically on the role of the OER in tamoxifen action during the mid 1970s so that there would be a better understanding of tamoxifen action in its target tissues, the mammary tu mour and uterus.[29–34]

At this time, we also made the observation that hydroxyl ated metabolites played an important role in the antioestro

genic and antitumour actions of tamoxifen.[35,36] We concluded that it was an advantage, but not a requirement, for tamoxifen to be metabolically activated to 4 hydroxytam oxifen. As a result of these studies, 4 hydroxytamoxifen be came the standard laboratory tool to study the molecular biology of antioestrogen action in vitro and in 1998 was used to crystallise the ligand binding domain of the OER with an antioestrogenic molecule.[37] The key to this accomplishment was that 4 hydroxytamoxifen has about a 100× higher binding affinity for the OER than tamoxifen.

## 5.    Tamoxifen for prevention? (Jordan VC. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. Eur J Cancer 1976;12:419–24)

In 1936, Profession Antoine Lacassagne suggested, based on his animal studies, that 'a therapeutic antagonist should be found to prevent the congestion of oestrone in the breast' so that breast cancer could be prevented.[38] Forty years later, the first experiment I was to complete with tamoxifen showed that just two injections of the 'antioestrogen' would almost completely prevent carcinogenesis in the rat mam mary gland.[2,39] I concluded that the mechanism was most likely blocking oestrogen action at the level of the OER in the mammary tissue and nascent tumour. These and subse quent studies[40–42] provided the scientific foundation for the eventual examination of the worth of tamoxifen to prevent breast cancer in high risk pre  and post menopausal wo men.[43–46] The key to tamoxifen's success in this application was a sustained duration of action and its ability to produce antitumour actions long after the therapy has stopped.[44,47]

## 6.    Long-term adjuvant tamoxifen therapy (Jordan VC, Allen KE. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur J Cancer 1980;16:239–51)

In the 1970s, the initial clinical studies of tamoxifen were fo cused entirely on its application as a treatment for metastatic breast cancer. The efficacy of tamoxifen was the same as that of high dose oestrogen therapy (diethylstilboestrol 15 mg dai ly), but the advantage of tamoxifen was fewer serious side ef fects.[13,48] The translation of the early laboratory findings with tamoxifen[1,2] to the treatment of advanced breast cancer showed an association between the efficacy of tamoxifen as an antitumour agent and OER status.[49] However, it was the transition from the use of tamoxifen as a palliative therapy to adjuvant therapy that was to have the greatest impact on survivorship and to establish tamoxifen as the gold standard for antihormonal therapy from 1980 to 2000.

The goal of adjuvant therapy is to destroy the micrometas tases that have spread around the body at the time of primary surgery. Early results with chemotherapy were extremely promising[50,51] and some significant improvements were noted once the overview analysis of worldwide randomised clinical trials was analysed and published.[52] However, the use of tamoxifen in this application was less readily accepted

because of the belief that tamoxifen was only a palliative therapy. As a prelude to the application of tamoxifen as an adjuvant therapy, I introduced the antioestrogen first to the Eastern Cooperative Oncology Group (ECOG)[53,54] and subsequently to the National Surgical Breast and Bowel Project (NSABP).[55] Early adjuvant clinical trials selected one year of adjuvant therapy[56–60] because of the fact that tamoxifen was effective in unselected patients with advanced breast cancer for about one year and there was a sincere concern that longer therapy would induce pre mature drug resistance. These beliefs were to change in the mid 1970s with the laboratory finding that long term antihormonal therapy was more effective than short term therapy.

Marc Lippman published an important paper in 1975 on the actions of tamoxifen in cell culture.[61] He demonstrated that oestradiol could reverse the action of tamoxifen to stop



Fig. 2 – Participants at a Breast Cancer Symposium in September 1977 at Kings College, Cambridge, England. The concept of extended adjuvant tamoxifen treatment was first proposed at this meeting. Clinical studies of a 1-year adjuvant tamoxifen were in place; regrettably, a decade later this approach was shown to produce little survival benefit for patients. In the insets (top), the author, who presented the new concept (bottom left); Professor Michael Baum, the session chairman who was about to launch the Nolvadez Adjuvant Trial Organization (NATO) 2-year adjuvant tamoxifen trial[95,96]; and (bottom right) Dr. Helen Stewart, who was a participant at the conference. She would initiate a pilot trial in 1978 and, led by Sir Patrick Forest, would later guide the full randomised Scottish Trial of 5 years' adjuvant tamoxifen treatment versus control in the 1980s.[97] Both clinical trials were later proven to produce survival advantages for patients. The concept of longer tamoxifen treatment producing more survival benefits for patients was eventually established indirectly by the Oxford Overview Analysis in 1992 and directly by the Swedish group led by Dr. Lars Rutqvist.[98]

cell replication and that tamoxifen could actually kill breast cancer cells at high concentrations. We decided to test the idea that tamoxifen was cytotoxic *in vivo* using the DMBA in duced rat mammary carcinoma model.

We reasoned that daily treatment with tamoxifen for a month in the rat would be equivalent to a year in a woman. Administration of DMBA (20 mg in 2 ml peanut oil po) to 50 day old female Sprague Dawley rats resulted in the develop ment of multiple mammary tumours in all animals about 150 d later.[62] The experimental approach we used was to administer different daily doses of tamoxifen for a month starting one month after DMBA administration. This design was to allow carcinogenesis to proceed following DMBA administration so that we could assess the effectiveness of tamoxifen to destroy the microfoci of deranged cells in the mammary tissue. This was as close as one could get to an endocrine adjuvant model in the 1970s.

Tamoxifen was compared with 4 hydroxytamoxifen be cause we had found it was the most potent antioestrogen then known[31]; at least 10 times more potent than tamoxifen. We chose to test 4 hydroxytamoxifen because we antici pated that it would be a more potent antitumour agent than tamoxifen. To our surprise, not only was 4 hydroxytamoxi fen not as effective as tamoxifen, but short term tamoxifen was unable to 'cure' animals. High doses of tamoxifen were superior to low doses of tamoxifen in reducing tumour num bers and controlled tumour appearance, but all animals eventually developed at least one tumour. Clearly, there was a link between dose and anticancer action, but it was because higher doses were cleared from the body more slowly and not that the higher dose was more active. Tamox ifen was acting as a tumouristatic agent the drug was effective as long as the drug was present to suppress tumour growth (Fig. 2).[3,63,64] We proved this concept experimentally by showing that antioestrogens were effective at controlling tumourigenesis as long as treatment was continued. Indeed, if tumours occurred during antioestrogen therapy, they would respond to a second antihormone therapy, in this case, oestrogen withdrawal following ovariectomy. We con cluded '*It was clear that antioestrogens do not destroy all the foci of hormone dependent tumour cells and long courses of therapy or the use of antihormonal methods e.g. ovariectomy are essential to control tumour growth*'.[3] This notion lead to the idea that longer was going to be better as a strategy to employ for adjuvant tamoxifen therapy and provided a scientific foun dation for the successful use of subsequent oestrogen depri vation, i.e. an aromatase inhibitor following 5 years of tamoxifen treatment.[65,66]

The overview analysis of randomised clinical trials that oc curs about every five years at Oxford has really revolutionised clinical thinking. The publications summarise treatment pro gress through the clinical trials mechanism. The clinical proof that longer tamoxifen therapy is better than shorter tamoxi fen therapy is most readily demonstrated in the OER positive pre menopausal patients. One year of tamoxifen was ineffec tive, but 5 years produced a dramatic effect on disease free survival and overall survival.[67] More importantly, tamoxifen produced a survival advantage for women, of a magnitude that would change the perception of endocrine agents as only palliative. The key to success was targeting women with the right tumour with the correct duration of treatment at the right stage.

## 7. Conclusion

What were the consequences of reinventing a failed contra ceptive ICI46,474[16] to become tamoxifen, the first targeted agent for the treatment of breast cancer[9]? The laboratory strategy of targeting OER positive tumours[1] with long term adjuvant therapy[3,64] ultimately resulted in the improved sur vivorship of hundreds of thousands of women [67,68] around the world. Indeed, the fact that tamoxifen is cheap and acces sible to under funded healthcare systems worldwide means that this form of targeted therapy continues to save lives. However, unlike the targeted therapies of today that usually have a single anticancer application, tamoxifen became the gold standard for the targeted therapy of all stages of breast cancer (including male breast cancer), the treatment of ductal carcinoma in situ,[69] a pioneering agent for the chemopreven tion of breast cancer in high risk women[45,70,71] and the lead compound for the new drug group, the SERMs.[72–76]

The extensive laboratory studies of tamoxifen and the re lated non steroidal antioestrogen LY156,758 (keoxifene) under taken as a prelude to initiating major trials in breast cancer prevention, described the pharmacology of SERMs that switch on and switch off target sites throughout the body. As an exam ple of the immediate translation of the discovery of SERM ac tion, tamoxifen was noted to block breast cancer growth but enhances the growth of endometrial cancer growth under lab oratory conditions.[77] This laboratory concept translated to im proved clinical care through awareness that tamoxifen increased the incidence of endometrial cancer in post meno pausal women treated for breast cancer. In another example of the application of SERMs, a failed breast cancer drug, keoxif ene, was reinvented[42,72,78] as raloxifene, the first SERM to be successfully used to treat osteoporosis with the beneficial side effect of preventing breast cancer indirectly.[79,80] Following rigorous testing in clinical trials,[81] raloxifene is now also avail able to prevent breast cancer in high risk post menopausal wo men. The overall result of 30 years of translational research in breast cancer prevention is that there are now two therapeutic options, tamoxifen and raloxifene, for women who choose to reduce their risk of breast cancer.[81,82] Thirty years ago there were no choices. Based on clinical testing, tamoxifen has a good risk benefit ratio in pre menopausal women[83] and raloxif ene has a better safety profile in post menopausal women.[81] It should be stressed, however, that raloxifene cannot be used to reduce breast cancer risk in premenopausal women.

Perhaps of greater significance is the fact that tamoxifen has become a pioneering agent to initiate new investigations in therapeutics. A study of the pharmacology of tamoxifen has been the catalyst to study the pharmacogenomics of tamoxifen which is redefining healthcare.[84] It appears that the specific metabolism of tamoxifen to a hydroxylated metabolite endoxifen is important for anticancer actions. This topic has recently been reviewed.[85] Finally, the importance of understanding the unique pharmacology of tamoxifen can be placed in perspective. In retrospect, tamoxifen could, in fact, be viewed as the lead compound that was essential to initiate the synthesis of a broad range of new SERMs for the treatment

of diseases as diverse as osteoporosis[86–92] and rheumatoid arthritis[93,94] and the subsequent extrapolation of the SERM concept to all members of the nuclear receptor superfamily.[76] The advances documented with targeting tamoxifen now of fer the promise of designing drugs to treat diseases previously thought to be impossible.

## Conflict of interest statement

None declared.

## Acknowledgements

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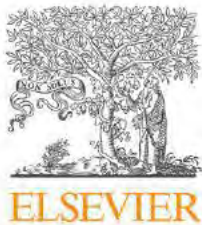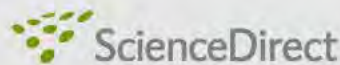
# Overexpression of CEACAM6 promotes migration and invasion of oestrogen-deprived breast cancer cells

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ABSTRACT

Carcinoembryonic antigen related cell adhesion molecule 6 (CEACAM6) is an intercellular adhesion molecule that is overexpressed in a wide variety of human cancers, including colon, breast and lung and is associated with tumourigenesis, tumour cell adhesion, invasion and metastasis. In this study, we showed that CEACAM6 was overexpressed in a panel of oestrogen receptor (ERα) positive human breast cancer cell lines (MCF 7:5C and MCF 7:2A) that have acquired resistance to oestrogen deprivation, and this overexpression was associated with a more aggressive invasive phenotype in vitro. Expression array analysis revealed that MCF 7:5C and MCF 7:2A cells overexpressed CEACAM6 mRNA by 27 fold and 12 fold, respectively, and were 6–15 times more invasive compared to non invasive wild type MCF 7 cells which expressed low levels of CEACAM6. Suppression of CEACAM6 expression using small interfering RNA (siRNA) completely reversed migration and invasion of MCF 7:5C and MCF 7:2A cells and it significantly reduced phosphorylated Akt and c Src expression in these cells. In conclusion, our findings establish CEACAM6 as a unique mediator of migration and invasion of drug resistant oestrogen deprived breast cancer cells and suggest that this protein could be an important biomarker of metastasis.

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## 1. Introduction

Carcinoembryonic antigen related cell adhesion molecule 6 (CEACAM6) is a glycosylphosphatidylinositol anchored cell surface protein that functions as a homotypic intercellular adhesion molecule.[1] It is overexpressed in a number of human malignancies including pancreatic cancer, gastrointestinal cancer and breast cancers[2,3], and increased levels of CEACAM6 are inversely correlated to the differentiation state of cancer cells. Previous studies have shown that CEACAM6 is overexpressed in pancreatic adenocarcinoma cells, and its overexpression is associated with greater in vivo metastatic ability and increased invasiveness and migration.[4,5] More recently, Poola and co workers[6] reported that the expression of CEACAM6 in atypical ductal hyperplasia was associated with the development of invasive breast cancer (IBC). Currently, however, the role of CEACAM6 overexpression in breast cancer migration and invasion is not known.

Invasion and metastasis are the hallmarks of cancer malignancy, and they are the primary cause of patient mortality during breast cancer progression.[7] Invasion refers to the ability of cancer cells to penetrate through the membranes that separate them from healthy tissues and blood vessels, and metastasis refers to the spreading of cancer cells to other parts of the body.[8] In order for a transformed cell to metastasize, it must first lose adhesion, penetrate and invade the surrounding extracellular matrix (ECM), enter the vascular system and adhere to distant organs.[8] These processes re

quire extensive alterations in gene expression profiles, including the down regulation of genes involved in cell anchorage and the up regulation of genes involved in cell motility and matrix degradation.[7,9,10]

Aromatase inhibitors (AIs) are anti oestrogen agents that suppress oestrogen production in peripheral tissues and breast tumours by inhibiting or inactivating aromatase, the enzyme which catalyses the conversion of androgens to oes trogens in post menopausal women.[11] Several randomized trials[12–15] have shown that third generation AIs are superior to adjuvant tamoxifen in terms of improved disease free sur vival and less side effects. Unfortunately, one of the conse quences of prolonged oestrogen deprivation/suppression is the development of drug resistance.[16,17] Previous studies have shown that acquisition of tamoxifen resistance in breast can cer cells is associated with a significant increase in motility and invasion[18,19] along with increased CEACAM6 expres sion[20]; however, it is unknown whether acquired resistance to oestrogen deprivation affects tumour cell migration and invasion and whether CEACAM6 plays a role in this process.

In this study, we investigated the role of CEACAM6 in cel lular migration and invasion of breast cancer cells that have acquired resistance to oestrogen deprivation. We found that CEACAM6 was significantly overexpressed in oestrogen de prived MCF 7:5C and MCF 7:2A breast cancer cells and that these cells were markedly more migratory and invasive than parental MCF 7 cells. Suppression of CEACAM6 expression by small interfering RNA (siRNA) completely reversed the invasive phenotype of MCF 7:5C and MCF 7:2A cells. E cad herin and β catenin were also significantly reduced in these cells. The mechanism of action of CEACAM6 appears to in volve, in part, the c Src and Akt signalling pathways.

## 2. Materials and methods

### 2.1. Reagents

17 Beta oestradiol was purchased from Sigma Chemical Co. (St Louis, MO); PP2 was purchased from EMD Biosciences Inc. (La Jolla, CA); LY294002 was purchased from Promega (Madison, WI); fulvestrant was obtained as a generous gift from AstraZeneca (Macclesfield, United Kingdom); Affymetrix Human Genome U133 Plus 2.0 Arrays were purchased from Affymetrix (Santa Clara, CA); foetal bovine serum (FBS), cell culture medium and other reagents were purchased from Invitrogen (Carlsbad, CA).

### 2.2. Cell lines and culture conditions

Wild type MCF 7 human breast cancer cells[21] were obtained from Dr. Dean Edwards (University of Texas, San Antonio, TX) and were maintained in fully oestrogenized medium (RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1× non essential amino acids and bovine insulin at 6 ng/mL (Sigma Aldrich, St. Louis, MO). MCF 7:5C[21–23] and MCF 7:2A[24] cells were clonally selected from parental MCF 7 cells following long term culture (>1 year) in phenol red free RPMI 1640 media containing 10% dextran coated charcoal stripped FBS (SFS).

### 2.3. RNA preparation and microarray hybridisation

Total RNA was prepared using the Qiagen RNeasy Mini kit. A DNase I digestion step was included to eliminate DNA con tamination. cRNA was generated, labelled, and hybridised to the Affymetrix Human Genome U133 Plus 2.0 Arrays by the Northwestern University Genomics Core (Chicago, IL). Arrays were washed, stained and scanned according to the direc tions detailed in the Affymetrix GeneChip® Expression Anal ysis Technical Manual.

### 2.4. Microarray data analysis

Assessment of data quality was conducted following default guidelines in the Affymetrixs GeneChip® Expression Analysis Data Analysis Fundamentals Training Manual. Data were ex tracted and normalised using *Affymetrix Microarray Suite* (MAS5.0) following recommended protocols for background and chip correction. Global scaling for average signal inten sity for all arrays was set to 500. Four biological replicates from each of the three cell lines were arrayed to determine consistent and reproducible patterns of gene expression. All but one array showed a high degree of reproducibility within a set of replicate hybridisations, leaving at least three array replicates per cell line for further analysis. Genes across all ar rays with an expression intensity <70 were removed. To elim inate genes with variable expression within a group of replicates, normalised gene intensity ratios (signal intensities divided by the median gene intensity all hybridisations) were derived, then the standard deviation of the log transformed normalised intensity ratios were calculated for each group of replicates. Genes with a standard deviation >0.15 were ex cluded. Lastly, to filter for genes with variable expression be tween cell lines, genes were retained that showed a standard deviation of >0.3. A total of 904 genes met the filtering criteria described and were examined by hierarchical clustering using resources available at TGen.[c] Uncentred Pearson's correlation with average linkage was used on $\log_2$ transformed data, with induced genes indicated in red and repressed genes in green. Random permutation analysis was performed as previously described [25] using 10,000 permutations. Genes with a $p$ value <0.01 and an alpha value <0.01 were used for gene ontology analysis.

### 2.5. Cell proliferation assay

Cell proliferation assay was performed as previously de scribed.[22] The DNA content of the cells was determined using a Fluorescent DNA Quantitation kit (Bio Rad Laboratories, Hercules, CA). For each analysis, three replicate wells were used, and at least three independent experiments were performed.

### 2.6. Western blot analysis

Western blot analyses were performed as previously de scribed.[22] Separated proteins were transferred onto nitrocel

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lulose membranes (Milllipore) and incubated overnight at 4 °C with the respective primary antibodies; CEACAM6 and CEA CAM5 (Signet Laboratories, Dedham, MA); ERα, N cadherin, β catenin, CXCR4, MMP9, E cadherin and CD44 (Santa Cruz Biotechnology, Santa Cruz, CA); fibronectin (Chemicon Inter national, Temecula, CA); c Src and p Src$^{Tyr529}$ (Biosource International, Carmarillo, CA); AKT and p AKT$^{Ser473}$ (Cell Sig naling Technology, Beverly, MA); and β actin (Sigma Chemical Co., St Louis, MO). Secondary antibodies conjugated to horse radish peroxidase (Santa Cruz Biotechnology) were used with an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL) to visualise the resolved proteins.

### 2.7. Quantitative real-time polymerase chain reaction (qRT-PCR) for ERα and CEACAM6

Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Ten micrograms of total RNA for each sample were converted to first strand cDNA using SuperScript III with a combination of random hexamers and oligo(dT) as primers (Invitrogen). Quantitative real time PCR assays were done as previously described[22] with the Taqman Universal or SYBR Green PCR Master Mixes and an ABI 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). The ERα forward and reverse primers were 5′ AAGAGGGTGCCAGGCTTTGT 3′ and 5′ CAGGATCTCTAGCCAGGCAC AT 3′, respectively. The ERα probe was 5′ [FAM] ATTTGACCCTCCATGATCAGGTCC ACC [TAMRA] 3′. The forward and reverse primers for CEA CAM6 were synthesised by Sigma Genosys (Sigma Aldrich). The sequences for CEACAM6 forward and reverse primers were 5′ GACGTTTGTGTGGATTGCTGGAACGC 3′ and 5′ TGCCACGCAGCCTCTAACC 3′, respectively. The reporter dye at the 5′ end of each probe was FAM and the quencher dye at the 3′ end was TAMRA. The 18S ribosomal RNA (18S rRNA) gene was used as an endogenous control to normalise for dif ferences in the amount of total RNA in each sample, 18S rRNA primers and probes were purchased from Applied Biosys tems. Relative expression of the target gene was calculated using the 2 delta CT method described previously[26] (Relative expression $2^{-\Delta CT}$; where $\Delta CT$ $C_{T\,(Target\,gene)} - C_{T\,(endogenous\,control\,gene)}$), where 18S rRNA is the endogenous control gene. To determine relative RNA levels within the samples, stan dard curves for the PCR were prepared by using cDNA from one sample and making twofold serial dilutions covering the range equivalent to 20 0.625 ng RNA (for 18S rRNA analy ses, the range was 4 0.125 ng).

### 2.8. Cell migration and invasion assays

Cell migration was measured in a Boyden chamber using Transwell filters obtained from Corning (Cambridge, MA). Cells ($1 \times 10^5$) in 0.5 mL serum free medium were placed in the upper chamber, and the lower chamber was loaded with 0.8 mL medium containing 10% SFS. Cells that migrated to the lower surface of filters were stained with Wright Giemsa solution, and five fields of each well were counted after 24 or 48 h of incubation at 37 °C with 5% $CO_2$. Three wells were examined for each condition and cell type, and the experi ments were repeated in triplicate. Cell invasion assay was

performed using the Chemicon cell invasion kit (Chemicon International, Temecula, CA) in accordance with the manu facturer's protocol. Cells ($1 \times 10^5$/ml) were seeded onto 12 well cell culture chamber using inserts with 8 μM pore size poly carbonate membrane over a thin layer of extracellular matrix. Following incubation of the plates for 48 h at 37 °C, cells that had invaded through the ECM layer and migrated to the lower surface of the membrane were stained and counted under the microscope in at least 10 different fields and photographed.

### 2.9. CEACAM6 siRNA-mediated gene knockdown

CEACAM6 specific siRNA (Silencer™ Predesigned siRNA; sense: GCCCUGGUGUAUUU UCAUtt, antisense: AUC GAAAAUACAC CAGGGCtg) (AM16704) and scramble sequence control siRNA (Silencer™ Negative Control siRNA) were pur chased from Ambion (Austin, TX). Transfection complexes were prepared in Opti MEM serum free medium (Invitrogen) by mixing 0.3 μL of siPORT NeoFX transfection reagent (Ambi on) and 10 nM CEACAM6 siRNA or negative control siRNA (Ambion). Cells ($9 \times 10^4$ cells per well) were reverse transfec ted in 12 well plates simultaneously with addition of trans fection complexes. The medium was replaced with phenol red free RPMI supplemented with 10% SFS 24 h after transfec tion and cultures were harvested for CEACAM6 protein and mRNA analyses.

### 2.10. Statistical analyses

Statistical analyses were performed using Microsoft Excel (Seattle, WA). Differences between groups were evaluated using Student's t test. Data were considered significant if $p < 0.05$.

## 3. Results

### 3.1. Characterisation of long-term oestrogen-deprived breast cancer cells

The growth of oestrogen deprived MCF 7:5C and MCF 7:2A cells is compared to parental MCF 7 cells in Fig. 1A. Both MCF 7:5C and MCF 7:2A cells grew robustly in the absence of oestrogen whereas MCF 7 cells grew minimally without oestrogen. The doubling times were 2.7, 3.4, and 6 d for MCF 7:5C, MCF 7:2A and MCF 7 cells, respectively. We also examined cell morphology changes associated with resis tance to long term oestrogen deprivation using phase con trast microscopy. Fig. 1B shows that MCF 7 cells grew as a uniform monolayer of tightly associated cells with limited cell spreading but distinct cellular boundaries, whereas oestro gen deprived MCF 7:5C and MCF 7:2A cells grew in a less uni form monolayer with cellular boundaries that were obscured. ERα mRNA and protein expression were also significantly in creased in MCF 7:5C and MCF 7:2A cells compared to MCF 7 cells and treatment with oestradiol or the pure anti oestrogen fulvestrant significantly down regulated its expression (Fig. 1C and D) in all three cell lines. Overall, these results show that oestrogen deprivation increases ERα expression and alters the morphology of MCF 7:5C and MCF 7:2A cells.
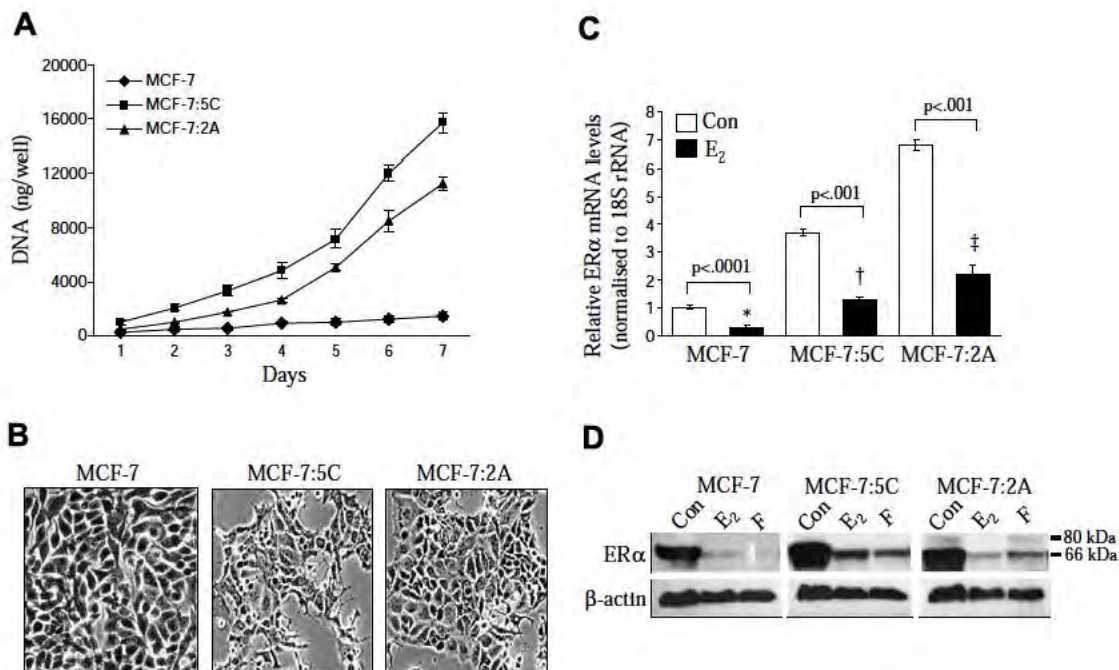
Fig. 1 – Characterisation of long-term oestrogen-deprived breast cancer cells. (A) For proliferation assays, cells were seeded in 24-well dishes (30,000 per well) in oestrogen-free RPMI media and total DNA was quantitated at the indicated time points. (B) Phase-contrast microscopy pictures of MCF-7, MCF-7:5C and MCF-7:2A cells. Images were produced by the Olympus DP-3030 camera and Olympus IX-70 software. Magnification, ×10. (C) ERα mRNA level was determined by quantitative RT-PCR. Relative expression of the target gene was calculated using the 2 delta CT method, where 18S rRNA was used as the endogenous control gene. All reactions were performed in triplicates and the error bar represents the standard deviation. (D) ERα protein levels were determined by immunoblotting with a specific ERα antibody. Cells were treated with 1 nM oestradiol or 1 μM fulvestrant for 48 h and 50 μg of protein lysates was analysed. β-Actin was used as a loading control.

## 3.2. Global gene expression profiles of oestrogen-deprived breast cancer cells

Transcriptional profiling of parental MCF 7 cells and oestrogen deprived MCF 7:5C and MCF 7:2A cells was performed using Affymetrix Human Genome U133 Plus 2.0 Array. Two dimensional hierarchical clustering was performed to analyse differences in gene expression patterns between MCF 7 cells and MCF 7:5C and MCF 7:2A cells. Data filtering identified 904 genes that were significantly altered between MCF 7:5C and MCF 7:2A cells and parental MCF 7 cells (Fig. 2A and Supplementary Fig. S1). The sample dendogram showed that MCF 7:2A cells and MCF 7 cells clustered more closely, whereas MCF 7:5C cells clustered on a more distant branch, suggesting that MCF 7:2A cells are more similar to parental MCF 7 cells than MCF 7:5C cells (Fig. 2A). In order to define cell signalling mechanisms that differed significantly between parental MCF 7 and MCF 7:5C and MCF 7:2A cells, random permutation weighted gene analysis was performed as described in Section 2. A comparison of MCF 7 expression data with that of MCF 7:5C and MCF 7:2A revealed that 4068 genes were highly differentially expressed (Supplementary Table 1). Gene Ontology analysis showed a significant number of genes associated with cell cycle control, proliferation, growth factor signalling, cell adhesion and motility and invasion. In particular, we found that CEACAM6 was overexpressed by 27 fold in MCF 7:5C cells and 12 fold in MCF 7:2A

cells (Fig. 2B), and it was highly weighted in our random per mutation analysis (p value < .0001) (Supplementary Table 1).

## 3.3. Oestrogen deprivation increases CEACAM6 expression and enhances migration and invasion of oestrogen-deprived breast cancer cells

To confirm our microarray data, CEACAM6 mRNA expression was determined by quantitative RT PCR. Fig. 3A shows that CEACAM6 mRNA was significantly upregulated in oestrogen deprived MCF 7:5C and MCF 7:2A cells compared with parental MCF 7 cells. Similarly, by Western blotting, CEACAM6 protein was undetectable in MCF 7 cells but was strongly expressed in MCF 7:5C and MCF 7:2A cells (Fig. 3B). Other invasion proteins such as CEACAM5, MMP9, CXCR4 and CD44 were also markedly elevated in MCF 7:5C and MCF 7:2A cells compared to MCF 7 cells (Fig. 3B). This finding is consistent with a recent study by Mackay and coworkers[27] which revealed that many genes associated with extracellular matrix remodelling were significantly upregulated following aromatase inhibitor treatment of primary breast tumours.

## 3.4. Oestrogen deprivation increases migration and invasion of breast cancer cells

Since MCF 7:5C and MCF 7:2A cells overexpressed several invasion genes, we next assessed the migratory and invasive

potential of these cells in vitro. Cell migration was measured using a modified Boyden chamber assay with 10% SFS as a che moattractant. As shown in Fig. 3C, MCF 7:5C and MCF 7:2A cells had the highest numbers of migrating cells compared to MCF 7 cells; a phenotype that correlated with CEACAM6 expression. Similar results were obtained when the different cell lines were tested for their ability to invade through mem branes coated with Matrigel. Fig. 3D shows that MCF 7:5C and MCF 7:2A cells had the highest number of invading cells, while MCF 7 cells were non invasive. The invasive ability of the cell lines was as follows: MCF 7:5C > MCF 7:2A > MCF 7.

### 3.5. CEACAM6 suppression inhibits invasion and migration of MCF-7:5C cells

To test the hypothesis that CEACAM6 is required for cell migration and invasion, we used siRNA to suppress CEACAM6 expression. MCF 7:5C cells were transfected with CEACAM6 specific or control (scrambled sequence) siRNA, and Western blot analysis was performed 72 h post transfection. Fig. 4A (top) shows that CEACAM6 protein was significantly sup pressed (75 85%) in MCF 7:5C cells transfected with the CEA CAM6 specific siRNA but not the control siRNA. siRNA suppression of CEACAM6 expression was also confirmed at the transcript level using qRT PCR at 48 h following transfec tion (Fig. 4A, bottom). To clarify the role of CEACAM6 in cell invasion, MCF 7:5C cells were pretreated with CEACAM6 siR NA or control siRNA for 48 h and invasion was measured over the subsequent 48 h. Fig. 4B shows that CEACAM6 siRNA al most completely reversed the invasiveness of MCF 7:5C cells, whereas control siRNA did not affect cell invasion. The inva siveness of MCF 7:5C cells was inhibited by nearly 80% when CEACAM6 expression was suppressed. A similar trend was observed for cell migration (data not shown). Suppression of CEACAM6 also significantly reduced phosphorylated Akt and phosphorylated c Src in MCF 7:5C cells (Fig. 4C). E cadherin and β catenin were also significantly reduced in MCF 7:5C and MCF 7:2A cells, whereas pAkt and N cadherin were sig nificantly upregulated in these cells compared to parental MCF 7 cells (Fig. 4D). Similar experiments performed in MCF 7:2A cells also showed a dramatic reduction (60%) in invasion following CEACAM6 suppression (data not shown).

### 3.6. Oestradiol down-regulates CEACAM6 expression and blocks migration and invasion of MCF-7:5C cells

We also examined whether CEACAM6 expression is hormon ally regulated in MCF 7:5C and MCF 7:2A cells. As shown in Fig. 5A and B, oestradiol completely down regulated CEA CAM6 mRNA and protein expression in MCF 7:5C and MCF 7:2A cells. This down regulation was an ERα mediated event since pretreatment with the anti oestrogen fulvestrant, which is known to degrade ERα[28,29], was able to reverse the inhibitory effect of oestradiol on CEACAM6 protein in both cell lines (Fig. 5B). Fulvestrant also completely counteracted the anti invasive effects of oestradiol in MCF 7:5C cells (Fig. 5C). Interestingly, oestradiol enhanced the invasiveness of parental MCF 7 cells (Fig. 5D) without significantly chang ing CEACAM6 protein level in these cells (Fig. 5B).
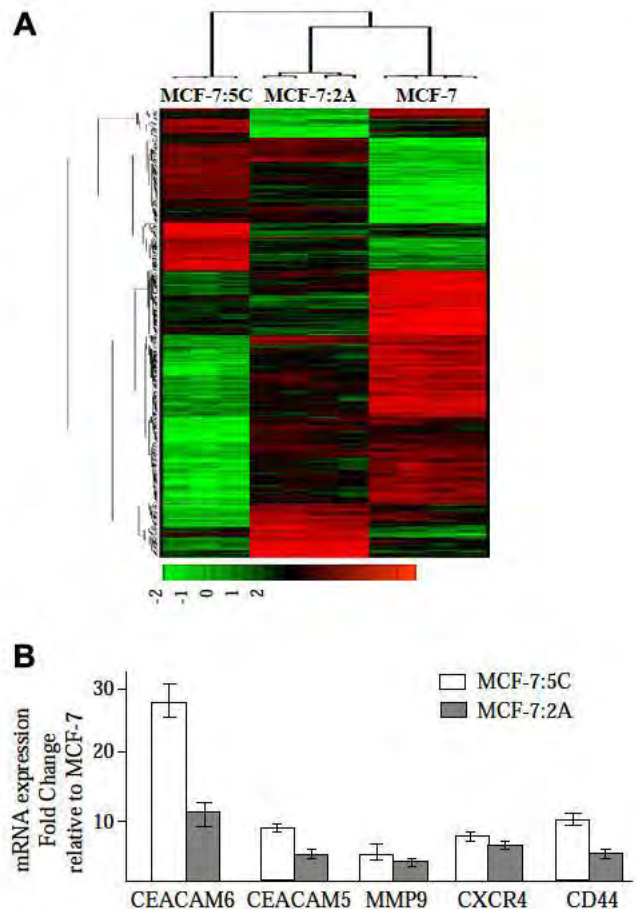
Fig. 2 – Overview of global gene expression patterns in wild-type MCF-7 cells and oestrogen-deprived MCF-7:5C and MCF-7:2A variant clones. (A) Unsupervised hierarchical clustering dendogram of 904 genes most differentially expressed across the three cell lines. Each row represents a single gene. Red, genes with high expression levels and green, genes with low expression levels. The similarities in the expression pattern amongst the three cell lines are presented as a "condition tree" on the top of the matrix. (B) Expression levels of invasion genes in MCF-7:5C and MCF-7:2A cells compared to parental MCF-7 cells, as identified by microarray analysis.

### 3.7. Inhibition of c-Src reduces the invasiveness of MCF-7:5C and MCF-7:2A cells

Previous studies have reported that CEACAM6 cross linking initiates c Src dependent cross talk between CEACAM6 and αvβ3 integrin, leading to increased ECM adhesion and inva sion.[30] We therefore determined c Src kinase activity in oest rogen deprived MCF 7:2A and MCF 7:5C cells by measuring phosphorylation of c Src at $Tyr^{529}$. Both MCF 7:5C and MCF 7:2A cells showed significantly elevated levels of phosphory lated c $Src^{Y529}$ compared to parental MCF 7 cells, and treat ment with the c Src kinase inhibitor PP2 significantly reduced the invasiveness of MCF 7:5C and MCF 7:2A cells (Supplementary Fig. S2). Inhibition of Akt phosphorylation using the PI3K inhibitor LY294002 also significantly reduced cell growth and invasion of these cells (Supplementary
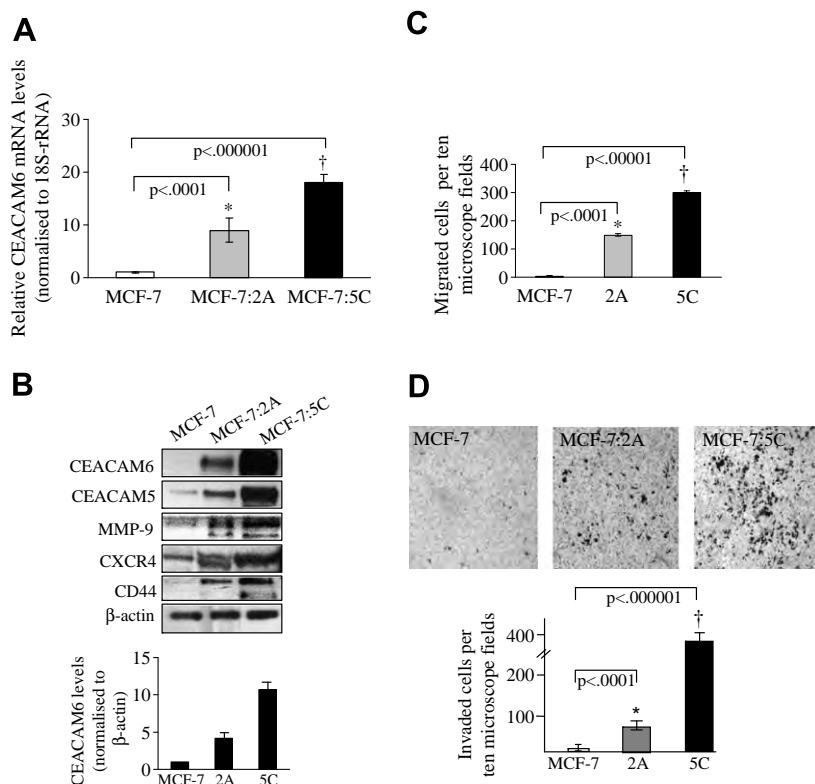
Fig. 3 – CEACAM6 promotes cell migration and invasion of oestrogen-deprived breast cancer cells. (A) CEACAM6 mRNA levels in parental MCF-7 cells and oestrogen-deprived MCF-7:5C and MCF-7:2A cells were measured by qRT-PCR. Relative expression of the target gene was calculated using the 2 delta CT method, where 18S rRNA was used as the endogenous control gene. All reactions were performed in triplicates, and the error bar represents the standard deviation. (B) Western blot analysis of CEACAM6 and other invasion proteins in MCF-7, MCF-7:5C and MCF-7:2A cells. The relative ratio of CEACAM6 was calculated by densitometry (bottom). The *bar graph* (bottom) depicts the averages of the data obtained from three individual experiments, and data are expressed as means ± SE. (C) Quantification of cells migrating across Transwell filters. (D) Cells that invaded through the Matrigel-coated transwells were fixed, stained, visualised at 20× magnification by light microscopy and photographed. Each panel represents an example of three replicates. Ten random fields were counted per insert at 20×.

Fig. S2), thus suggesting an important role for the c Src and Akt signalling pathways in invasion.

## 4. Discussion

Despite advances in detection and treatment of metastatic breast cancer, mortality from this disease remains high be cause current therapies are limited by the emergence of ther apy resistant cancer cells. In this study, we showed that oestrogen deprivation significantly increased the motility and invasiveness of two ERα positive human breast cancer cell lines that have acquired resistance to oestrogen depriva tion, and that these cells overexpressed the invasive gene CEACAM6. Furthermore, knockdown of CEACAM6 expression completely inhibited the invasiveness of MCF 7:5C and MCF 7:2A cells and caused a reduction in phosphorylated c Src and pAkt expression. A significant reduction in E cadherin and β catenin was also observed in MCF 7:5C and MCF 7:2A cells compared to parental MCF 7 cells. To our knowledge, this study is the first to demonstrate a critical role for CEA CAM6 in migration and invasion of breast cancer cells that have acquired resistance to oestrogen deprivation.

Previous studies have reported that overexpression of CEACAM6 in pancreatic adenocarcinoma cells is associated with enhanced cellular invasiveness and increased meta static potential *in vivo*, and that this effect is completely attenuated by suppression of CEACAM6 expression.[4] Recently, Scott and coworkers[20] reported that CEACAM6 was upregu lated by 20 fold in tamoxifen resistant MCF 7 cells compared to tamoxifen sensitive cells, and that hormone sensitivity could be partially restored in the tamoxifen resistant cells by siRNA silencing of CEACAM6. This *in vitro* data were sub stantiated in clinical breast cancer where it was demon strated that CEACAM6 was overexpressed in primary breast tumours that subsequently relapsed following adjuvant tamoxifen and in a multivariate analysis, only CEACAM6 re mained a significant predictor of recurrence.[31] These findings are consistent with our present study which shows that CEA CAM6 is significantly upregulated in oestrogen deprived breast cancer cells that have acquired resistance to oestrogen suppression, and knockdown of CEACAM6 expression re verses the invasive phenotype of these cells. The fact that CEACAM6 is identified independently in two model systems using endocrine agents with distinct modes of action sug gests that it may play an important role in endocrine resis
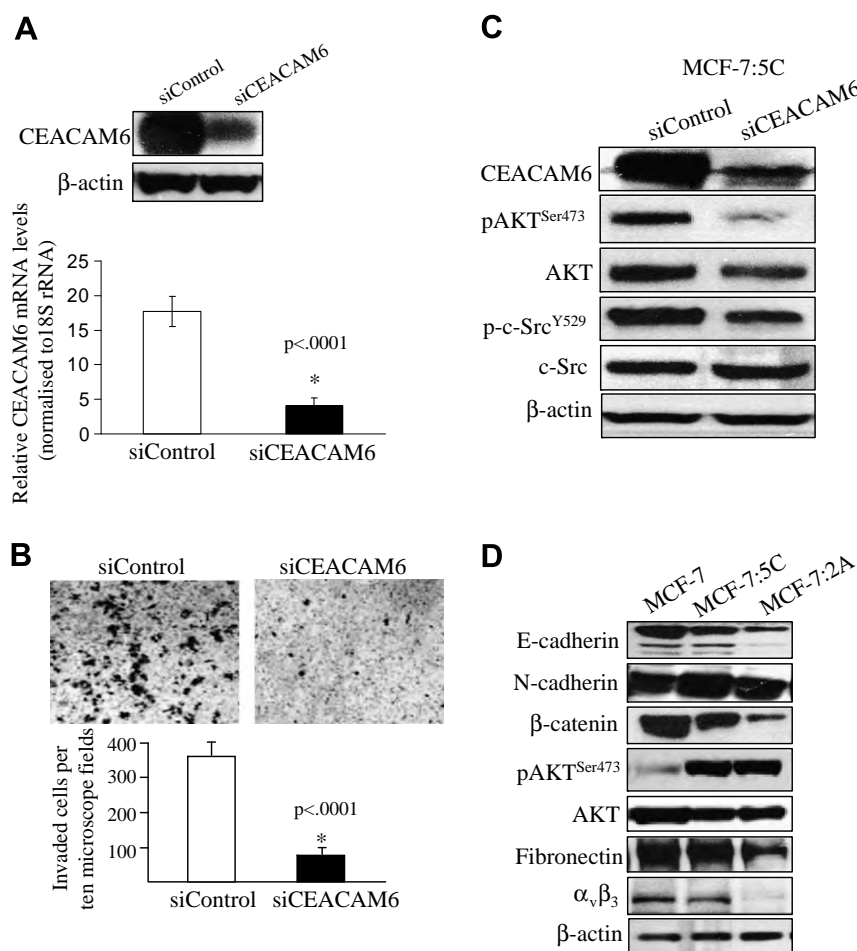
Fig. 4 – CEACAM6 suppression completely blocks invasion of MCF-7:5C breast cancer cells. (A) siRNA-mediated gene knockdown of CEACAM6 was verified by Western blot (top panel) and qRT-PCR (bottom panel). For qRT-PCR experiments, relative expression of CEACAM6 gene was calculated using the 2 delta CT method, where 18S rRNA was used as the endogenous control gene. All reactions were performed in triplicates and the error bar represents the standard deviation. (B) Matrigel invasion assay of siControl and siCEACAM6-transfected MCF-7:5C cells. (C) Immunoblot analysis of MCF-7:5C cells transfected with CEACAM6 siRNA or control siRNA for 72 h. β-Actin was used as a loading control. (D) Western blot analyses of E-cadherin, β-catenin, N-cadherin, Akt and pAKT protein expression in MCF-7, MCF-7:5C and MCF-7:2A cells.

tance. Currently, the mechanism by which CEACAM6 facilitates invasion is not fully understood. However, there is evidence that CEACAM6, along with other GPI anchored proteins, is capable of modulating the activity of intracellular tyrosine kinases such as c Src.[32,33] In particular, studies by Duxbury and coworkers[30,34] showed that c Src activity was increased in CEACAM6 overexpressing BxPC3 human pancreatic cancer cells and decreased following suppression of CEACAM6 expression, and that inhibition of c Src activity significantly suppressed CEACAM6 mediated cellular invasiveness. We found that phosphorylated c Src was significantly elevated in MCF 7:5C and MCF 7:2A cells, and that suppression of CEACAM6 expression reduced its level in these cells. Pharmacological blockade of c Src using the Src tyrosine kinase inhibitor pyrazolopyrimidine (PP2) also inhibited the invasiveness of MCF 7:5C and MCF 7:2A cells. In addition, we found markedly elevated levels of phosphorylated Akt ser473 in MCF 7:5C and MCF 7:2A cells, which were dramatically reduced following CEACAM6 suppression. Akt is a serine/threonine protein kinase that mediates cell survival, proliferation[35,36], tumour cell migration and invasion and metastasis,[37] and previous studies have shown that c Src activates the PI3K/Akt signalling pathway.[38] Thus, it is possible that activation of both c Src and Akt might play a role in mediating CEACAM6 induced migration and invasion.

The epithelial to mesenchymal transition (EMT) plays a key role in metastasis and is characterised by the conversion of epithelial cancer cells to a more motile phenotype that facilitates invasion. A critical molecular feature of EMT is the down regulation of E cadherin,[39] a cell adhesion molecule present in the plasma membrane of most normal epithelial cells. E cadherin acts de facto as a tumour suppressor inhibiting invasion and metastasis and is frequently repressed or degraded during transformation. In our study, E cadherin and β catenin were significantly decreased, whereas N cadherin was markedly increased in invasive MCF 7:5C and MCF 7:2A cells compared to non invasive MCF 7 cells. In addition, our cell morphology studies showed EMT like changes in MCF
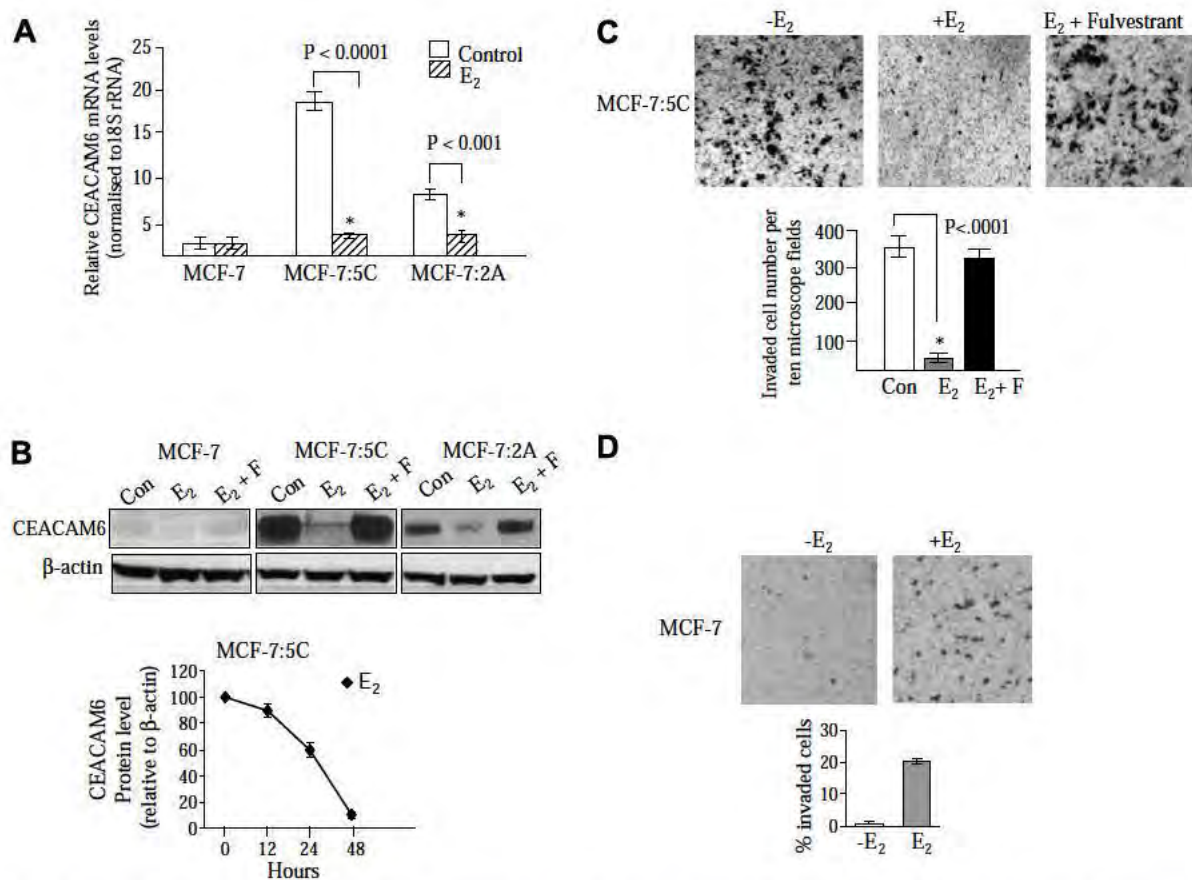
Fig. 5 – 17β-Oestradiol suppresses CEACAM6 expression and blocks invasion of oestrogen-deprived breast cancer cells. (A) Quantitative RT-PCR analyses of CEACAM6 mRNA expression in MCF-7:5C and MCF-7:2A cells following treatment with 1 nM oestradiol ($E_2$) for 48 h. Expression levels were internally normalised to the housekeeping gene 18S rRNA (error bars, SE) (B) Western blot analysis of CEACAM6 protein expression in MCF-7, MCF-7:2A and MCF-7:5C cells. *Line graph* shows the time-dependent effect of $E_2$ on CEACAM6 protein level in MCF-7:5C cells. (C) Invasion of MCF-7:5C cells is blocked by $E_2$ but not the pure anti-oestrogen fulvestrant. Invasion assay was performed as previously described in Fig. 3. (D) Effect of oestradiol on the invasiveness of wild-type MCF-7 cells. Each panel represents an example of three replicates.

7:5C and MCF 7:2A cells compared to MCF 7 cells. A variety of signal transduction pathways impinge on the regulation of E cadherin levels or subcellular distribution. In particular, Akt/PKB has been shown to repress transcription of the E cad herin gene, which leads to conversion of epithelial cells into invasive mesenchymal cells.[40] We have found that MCF 7:5C and MCF 7:2A both cells overexpress phosphorylated Akt, and gene ontology analysis of expression data obtained for MCF 7:5C and MCF 7:2A cells reveals that the P13K/Akt sig nalling pathway is significantly ($p$ 0.002) altered compared to parental MCF 7 cells.

In conclusion, we have identified CEACAM6 as a critical gene in the regulation of migration and invasion of breast cancer cells that have acquired resistance to oestrogen depri vation. Since aromatase inhibitors are now considered the standard of care for the hormonal treatment of early breast cancer in postmenopausal women, this finding has important clinical implications for these patients because it suggests that extended use of aromatase inhibitors may potentially lead to the development of metastatic disease. CEACAM6 can thus serve as a powerful predictor of future recurrence and may also represent a promising new therapeutic target for breast cancer.

## Conflict of interest statement

None declared.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2008.05.016.

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# Chapter #

# Selective Estrogen Modulators as an Anticancer Tool:
## Mechanisms of Efficiency and Resistance

**Surojeet Sengupta and V. Craig Jordan***

## Abstract

The majority of breast cancers are estrogen receptor (ER) positive and depend on estrogen for growth. Therefore, blocking estrogen mediated actions remains the strategy of choice for the treatment and prevention of breast cancer. The selective estrogen receptor modulators (SERMs) are molecules that block estrogen action in breast cancer but can still potentially maintain the beneficial effects of estrogen in other tissues, such as bone and cardiovascular system. Tamoxifen, the prototypical drug of this class has been used extensively for the past 30 years to treat and prevent breast cancer. The target of drug action, ERs alpha and beta, are the two receptors which are responsible for the first step in estrogen and SERM action. The SERM binds to the ERs and confers a unique conformation to the complex. In a target site which expresses antiestrogenic actions, the conformation of the ER is distinctly different from estrogen bound ER. The complex recruits protein partners called corepressors to prevent the transcription of estrogen responsive genes. In contrast, at a predominantly estrogenic site coactivators for estrogen action are recruited. Unfortunately at an antiestrogenic site such as breast cancer, long term SERM therapy causes the development of acquired resistance. The breast and endometrial tumor cells selectively become SERM stimulated. Overexpression of receptor tyrosine kinases, HER-2, EGFR and IGFR and the signaling cascades following their activation are frequently involved in SERM resistant breast cancers. The aberrantly activated PI3K/AKT and MAPK pathways and their cross talk with the genomic components of the ER action are implicated in SERM resistance. Other down stream factors of HER-2 and EGFR signaling, such as PI3K/AKT, MAPK or mTOR pathways has also been found to be involved in resistance mechanisms. Blocking the actions of HER-2 and EGFR represent a rational strategy for treating SERM resistant phenotypes and may in fact restore the sensitivity to the SERMs. Another approach exploits the discovery that low dose estrogen will induce apoptosis in the SERM resistant breast cancers. Numerous clinical studies are addressing these issues.

## Introduction

Selective estrogen receptor modulators (SERMs) are molecules which bind to estrogen receptors (ERs) and confer either estrogen-agonistic (estrogen-like) or estrogen-antagonistic (antiestrogen-like) actions in various estrogen target tissues and cells. In other words, the same SERM molecule can be estrogen agonistic in some tissues, as well as estrogen antagonistic in others, in the same organism at the same time. This pharmacology is unique and has allowed the SERMs

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to be not only valuable tools to dissect the subcellular action of estrogen but also has opened the door to important therapeutic applications. However, SERMs did not appear suddenly as a new drug group but were originally referred to as nonsteroidal antiestrogens[1] that have continuously evolved and been evaluated for different clinical application during the past 50 years.

Nonsteroidal antiestrogens were originally investigated as agents to modulate reproductive functions.[2] They were effective as post coital contraceptives in rats[3] but actually induced ovulation in subfertile women.[4] The failure of antiestrogen to become antifertility agents throughout the 1960's resulted in a decline in interest by the pharmaceutical industry in developing the drug group. Nevertheless, the molecules were of pharmacological interest and became important tools in endocrine research to decipher the actions of estradiol (Fig. 1). As a drug group, the nonsteroidal antiestrogens were noted to block estrogen binding to its target tissues e.g., uterus, vagina and some breast cancers[5-7] because they were competitive inhibitors of estradiol binding to ER.[8,9]

One compound ICI 46,474 was studied extensively because fashions in research changed significantly during the 1970s. There was a new focus on cancer research which, in this case, built on the prior experience with reproductive endocrinology.[10] ICI 46,474, the failed contraceptive was reinvented to become tamoxifen (Fig. 1), the first antiestrogen for the treatment of breast cancer.[11] This in turn caused an evaluation of the molecular mechanisms of its antitumor action. During 1970's a treatment strategy was developed in the laboratory so that tamoxifen was subsequently targeted to the patients with ER positive tumors, administered as a long term adjuvant therapy in early stage disease which resulted in a significant advance in cancer therapy with survival advantages for hundreds of thousands of patients.[12]

In the laboratory, the discovery that tamoxifen needed to be hydroxylated to 4-hydroxytamoxifen to achieve high binding affinity for the ER[13,14] created an important laboratory tool to examine antitumor actions in vitro, to study structurefunction relationships[1,15] and ultimately to discover the actual molecular mechanisms of antiestrogen action at the ER level.[16] Overall the SERMs have played a pioneering role in cancer treatment both as laboratory tools and targeted agents in cancer
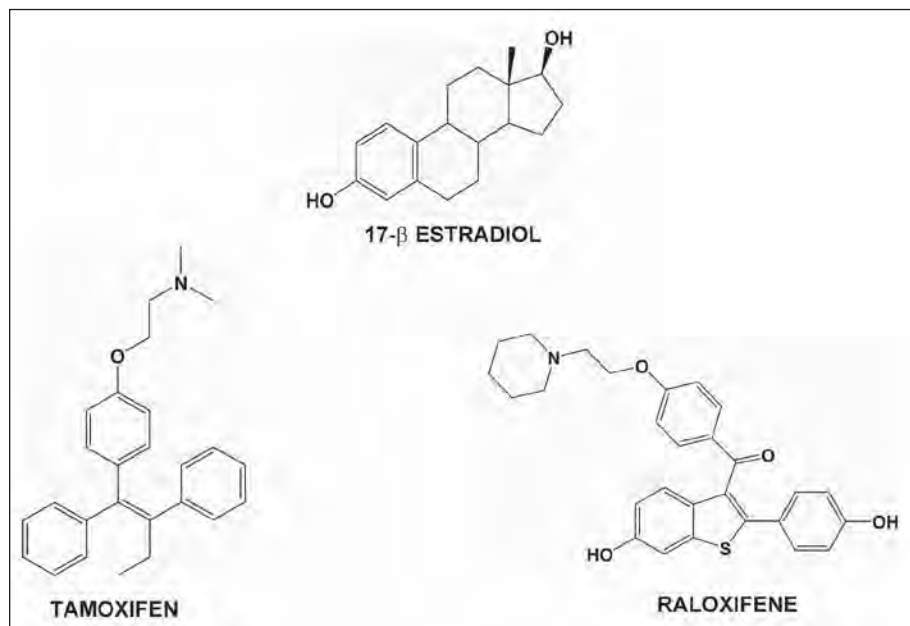
Figure 1. Chemical structures of 17-β estradiol, tamoxifen and raloxifene.

therapeutics. This chapter will trace their continuing development and current role in deciphering the complex signaling pathways that occur with the evolution of antihormonal drug resistance.

## Estrogen, Tamoxifen and Cancer

As early as 1896, Dr. George Thomas Beatson noted that ablation of the ovarian stimulus (estrogen) restricted the growth of breast cancers.[17] Unfortunately only limited numbers of the breast cancer responded to the ablative surgery. More than 50 years later, the studies by Elwood Jensen,[18] that initially defined the target site specificity of estrogen action, helped further in understanding the requirement of the ER for the estrogen dependent growth of breast cancers.[19] The potential of tamoxifen (known as an anti-estrogen, at that time) to be used as an anti-breast cancer agent was recognized when it was reinvented from a failed contraceptive to become the first targeted drug for the treatment of breast cancer (see above).[11] Numerous studies using laboratory animals demonstrated the anti-tumor effects of tamoxifen. Early studies using a carcinogen-induced rat mammary tumor model revealed that tamoxifen was able to inhibit the growth as well as the tumor initiation.[20-24] However, long term therapy was stated to be the correct clinical strategy for the adjuvant treatment of breast cancer.[25,26] Similar findings were subsequently noted in xeno-transplanted ER positive breast cancer cells in the athymic (immuno-deficient) mice model. Tamoxifen was able to inhibit the estrogen-induced growth of the ER expressing breast tumors (MCF7 and ZR75) but not of ER negative (MDA-MB 231) tumors.[27,28] Overall these studies clearly indicated the anti-tumor effects of tamoxifen in ER positive breast cancers. The knowledge from the laboratory experiments, that tamoxifen could be used as a therapeutic agent to treat ER positive breast cancers, were successfully translated to clinical trials.[29,30] An early overview study combining 40 adjuvant tamoxifen trials noted highly significant benefits in both disease-free and overall survival.[31] A subsequent overview of randomized trials relevant to tamoxifen indicated that longer (5 years) duration treatments with tamoxifen are beneficial than shorter (1-2 years) treatments. Significant reduction in mortality was also observed with 5 years of treatment than shorter treatments.[12] Unfortunately treatment duration more than five years do not produce further benefits,[32] however, effective continuing reduction in breast cancer recurrence is noted for more than a decade after the termination of tamoxifen therapy.[12,33] The clinical trials for tamoxifen as an adjuvant therapy for breast cancer also revealed that 5 years of tamoxifen therapy reduces the recurrence of breast cancer and also the incidences of contralateral second primary breast tumors by fifty percent.[12,34] This led to the possibility that tamoxifen has potential as a chemo-preventive agent. However, the chemosuppresive actions of tamoxifen was already established earlier in experiments done in laboratory animals.[20,35] Several studies have now established that tamoxifen can significantly reduce the number of ER positive breast cancers in high risk group of both pre and post-menopausal women,[33,36-39] and is currently in use for therapeutic prevention of ER positive breast cancers in high risk population.

The idea that SERMs could be multifunctional medicines was based on the laboratory observations that a failed breast cancer drug keoxifene[40] (LY156758) actually maintained bone density in ovariectomized rats[41] and the same doses prevented mammary cancer in rats.[42] Most importantly, keoxifene was less estrogenic than tamoxifen in the rodent uterus[43] and was shown less active at stimulating human endometrial cancer growth in laboratory animals.[44] The publication of the idea[35,45] that nonsteroidal compounds of the same class as tamoxifen could be used to prevent osteoporosis in postmenopausal women but prevent breast cancer at the same time directly led to the subsequent re-examination of the pharmacology of keoxifene and the renaming of the compound into raloxifene (Fig. 1). The clinical investigation that a SERM could be used to prevent osteoporotic fractures but at the same time reduce the incidence of breast cancer[46] created a new dimension in chemoprevention.[47] Raloxifene was advanced for testing against the veteran tamoxifen to reduce breast cancer incidence in high risk postmenopausal women in the study of tamoxifen and raloxifene or STAR trial. Recent reports[48] demonstrate that raloxifene is equally effective as tamoxifen in preventing breast cancers in post-menopausal women. The study also showed lower incidence of endometrial cancer associated with raloxifene treatment than in case of tamoxifen.

Therefore, the clinical foundation to discover the ideal SERM has now been established. The SERM should prevent breast and endometrial cancer but increase bone density and reduce fractures. The challenge of molecular medicine for the future is to decipher the endocrine mediated control mechanisms for reversing or slowing the development of atherosclerosis, reducing hot flashes and defining the importance of estrogen regulated CNS function. To achieve these goals there is now a focused effort to understand the molecular modulation of estrogen action using SERMs as laboratory tools in estrogen target tissues and to understand SERM-stimulated drug resistance to optimize cancer control.

## Molecular Mechanism of SERM Action

Mechanism of SERM action depends upon several factors. Essentially, SERMs bind to ERs α and/or β subtypes and confer a unique conformation to the ER. The complex further recruits coregulators and other accessory proteins at the estrogen-responsive elements of the promoters of specific genes to activate or repress transcription.[49] To completely understand the individual roles of these factors, we will discuss them separately.

### Estrogen Receptors

Two sub-types of ERs α and β are responsible for the estrogen or SERM mediated effects. Different binding affinities of SERMs to these receptors and differential expression of these two sub-types in various target cells may account for selective modulation in some tissues.[50] In addition, hetero-dimerized ERs α and β may induce unique effects on estrogen- and tamoxifen-dependent gene expression.[51] A recent report also indicates that ER β mediates the effects on ER α induced transcription in ER positive breast cancer cells.[52]

Structurally, ER protein can be subdivided into six domains based on the function controlled by that region. The A/B domain contains one of the two transcriptional activation functions (AFs), known as AF1 which is largely involved in estrogen-independent activation of transcription. Another activation function domain, AF2, is located in the E domain which also harbors the ligand binding domain (LBD) and is involved in estrogen/ligand-dependent activation.[53] The structural studies of LBD of ERs α and β complexed with a SERM reveal that reorientation of the AF2 helix (helix 12) after the binding of the SERM to the hydrophobic pocket of the LBD and the interaction of amino acid asp351 of ERα with the alkylaminoethoxyphenyl side chain of tamoxifen are crucial for the corepressor recruitment to the surface of SERM-receptor complex.[16,54,55] Due to the usage of different mutants of ERα for the amino acid asp351 it is known that shielding and neutralization of asp351 by the side chain of raloxifene is critical in defining the antiestrogenicity of this SERM.[56] The involvement of the asp351 is further exemplified by changing the aspartate to glycine which abolishes the estrogen-agonist activity of tamoxifen, while retaining its antagonistic property.[57] AF2 region of the agonist-bound receptor is particularly important for the interactions of steroid receptor coactivators (SRCs 1-3) via the interacting amino acid motif LxxLL. Recruitment of these co-activator(s) to the promoters of estrogen responsive genes is also responsible for facilitating the activation of transcriptional machinery by chromatin remodeling. Additionally, SERMs may also show differential AF1 activity mediated by corepressor binding.[58] Using ERE-reporter constructs, it has been shown that AF1 domain of ERα is actively involved in agonist-induced gene expression whereas AF1 domain of ER β is involved very weakly.[59]

The activated ER binds to the specific estrogen responsive elements (ERE), found within the promoter region of responsive genes. Significantly, the nature of these DNA sequences also influences the recruitment of the coregulator proteins to the ER at the promoters. Using various ERE containing DNA sequences, it has been found that liganded ER α and β regulate the interaction of the coregulators depending upon the type of ERE, to which the receptor is bound.[60]

### Coregulators

Interaction of particular coregulators (co-activators and corepressors) with the liganded estrogen receptors modulates the transcription of the responsive genes. Around 200 coactivators are currently known, which are associated with 48 nuclear receptors.[61] The coactivators undoubtedly

play defining roles in the activity of SERMs by cell or tissue specific expression pattern of genes. Studies have indicated that the relative abundance of a co-activator, SRC1 (steroid coactivator 1) in uterine cells is responsible for the agonistic activity of tamoxifen in those cells, whereas tamoxifen acts as an estrogen antagonist in breast cancer cells where the SRC1 levels are low.[62] However, raloxifene, another related SERM, does not recruit SRC-1 even in the uterine cells,[62] underscoring the fact that the SERM induced conformation of estrogen receptor is crucial for the interaction of coregulators. Consistent with these findings, earlier studies have reported tamoxifen-induced growth of endometrial cancer cells but not of breast cancer cells in athymic mice[63] and also that raloxifene (keoxifene) is less estrogenic to endometrial cancer cells.[44] These finding translate to clinical experience.[48] Furthermore, SERMs can also increase the stability of the co-activators (SRC1 and SRC3) and thereby enhance the transcriptional capability of other nuclear receptors.[64] In addition to transcriptional regulation, relative abundance and stability of co-activators, post-translational modifications particularly, different phosphorylation and sumoylation states of the co-activators can also drastically influence the capacity to interact with ER and other members of the transcriptional complex and regulate the gene activation.[65,66]

Corepressors proteins, on the other hand are functional counterparts of co-activators, which are associated with transcriptionally inactive promoters and thus help repress the expression of genes.[67] There are fewer corepressors known than the co-activators. In the case of ER, the co-repressors are known to interact with the unoccupied and antagonist bound receptor. Nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) are the two most extensively studied corepressors in connection with ER. The ER bound to raloxifene or 4-hydroxytamoxifen (a potent antagonist metabolite of tamoxifen) is known to recruit NCoR and SMRT to the promoters of estrogen responsive genes and repress transcription.[62,68,69] It has been shown that inhibition of NCoR or SMRT by using antibodies can enhance the agonistic property of 4-hydroxytamoxifen.[70] Moreover, using fibroblasts from NCoR null mice, 4-hydroxytamoxifen was shown to be relatively potent ERα agonist.[71] The critical role of NCoR and SMRT in 4-hydroxytamoxifen-induced arrest of cell proliferation of ERα positive breast cancer cells was illustrated when 4-hydroxytamoxifen-stimulated cell cycle progression was noted in the breast cancer cells deficient in NCoR and SMRT.[72] However this study also found that not all estrogen responsive genes were activated by 4-hydroxytamoxifen in NCoR and SMRT deficient cells, clearly indicating that other molecules may also be important in SERM-induced repression of estrogen responsive genes. Indeed, there are several other corepressor proteins known for ER. Metastasis associated protein 1 (MTA 1) is a corepressor found to mediate the ER transcriptional repression.[73] Another corepressor, known as repressor of estrogen action (REA) was able to potentiate the inhibitory effects of anti-estrogens including 4-hydroxytamoxifen. It was also found that REA interacted with ER and competed with the co-activator SRC1 for binding to the estrogen bound ER.[74,75] This again emphasizes the fact that the relative levels of coregulators may be important in deciding the outcome of the SERM action. The proteasomal regulation of NCoR is another factor which may influence the SERM action. Degradation of NCoR by 26S proteasome is known and is mediated by seven in absentia homologue 2 (Siah2).[76] Interestingly, estrogen mediated up-regulation of Siah2 in ER positive breast cancer cells has been implicated in proteasomal degradation of NCoR and subsequent de-repression of NCoR regulated genes.[77]

In addition to acting as a "transcriptional adapter" between the receptors and the transcriptional machinery, the coregulator itself or its complex possess various enzymatic activities such as acetylation, phosphorylation, methylation or deacetylation by which they are able to modify the local chromatin structure such as to make the environment conducive for gene expression or repression. Intrinsic histone acetyl transferase activity was found to be associated with co-activator SRC1 which helps in the activation of transcriptional expression.[78] In contrast, the 4-hydroxytamoxifen bound ER complex which recruits the corepressors NCoR and SMRT is associated with histone deacetylases and other chromatin modifying enzymes. The deacetylase activity promotes transcriptional repression.[62,79] Interestingly, another enzyme in the coactivator complex, CARM1 (coactivator associated arginine methyltransferase 1) has recently been implicated in modifying

the coactivator itself and inducing the degradation of the complex.[80] This suggests the ability of the enzymes in the complex to modify other proteins of its own complex apart from modification of the chromatin.

## Evolution of SERM Resistant Breast Cancers

The preventive and therapeutic efficacy of SERMs for breast cancers is limited by the development of resistance for the SERMs. Initially, the development of SERM resistance was considered as overgrowth of ER negative cell population, over the growth arrested ER positive cells, by the antiestrogen (SERM) treatment.[81] However, we now know that there are various forms of SERM resistant breast cancer and studies of these resistant forms have led to novel therapeutic approaches. In general terms, SERM resistant breast cancers can be divided into two categories (a) de novo resistance and (b) acquired resistance. De novo resistance is defined as ER positive breast cancers which are nonresponsive to SERM therapy from the very beginning. De novo resistance can be demonstrated in the laboratory when ER positive MCF-7 breast cancer cells are stably transfected with the HER-2/neu gene. Tumors form very rapidly even during tamoxifen treatment.[82] Acquired resistance, on the other hand are those ER positive breast cancers which initially respond to SERM therapy, but do not continue to respond during long term therapy[81] (Fig. 2). This concept is illustrated in the laboratory if wild type MCF-7 breast cancer cells are inoculated into ovariectomized athymic mice and treated with tamoxifen. Initially most tumors do not grow but some tumors start to grow in the presence of the antiestrogen after about a year. If the growing tumors are transplanted into other athymic mice they will grow in response to either estrogen or tamoxifen.[83] Functional ER expression is still maintained in these SERM resistant cells. SERM resistance is unique because when the SERM is complexed with ER there is SERM stimulated growth. Examination of this form of SERM resistance in the clinic demonstrates that SERM resistant tumors can still respond to fulvestrant, a pure ER antagonist or the aromatase inhibitors which block the peripheral synthesis of estrogen in postmenopausal women.[84] This form of drug resistance i.e., SERM stimulated growth is referred to as phase I drug resistance (Fig. 2). Models for tamoxifen and raloxifene resistance are well described in the literature.[83,85]

## Mechanism of SERM Resistance

Although the precise molecular mechanism for the SERM resistance is not completely understood, several genomic and extra-genomic factors are being shown to be involved in imparting resistance to SERMs or play a role in SERM induced growth of breast cancer cells. However, it is highly unlikely that any one particular mechanism is responsible for the SERM resistance in all patients. It could be possible that a combination of several factors may be responsible for the SERM resistance but for the sake of clarity these factors are discussed here individually.

### Role of Epidermal Growth Factor Receptors (EGFRs) in SERM Resistant Breast Cancers

Signaling cascades originating from the cell surface of the cancer cells may drastically influence the genomic actions mediated by ER. One of the most prominent and well studied signaling pathway is the EGFR2, also known as HER-2/neu. HER-2, a receptor tyrosine kinase, is a member of the EGFR family and its amplification or overexpression is frequently associated with an aggressive phenotype of cancers.[86-88] Indeed, overexpressing HER-2 in ER positive MCF-7 breast cancer cells prevents the cells from responding to tamoxifen.[82,89] The mechanism by which HER-2 overexpression confers tamoxifen resistance and switches tamoxifen bound ER to an agonistic configuration has recently been described[90] (Fig. 3). An increased cross-talk between HER-2 and estrogen signaling pathways coupled with high SRC3 levels are responsible for subverting the ability of the tamoxifen bound ER to recruit corepressors. Instead the tamoxifen ER complex recruits coactivator SRC3.[90] Consistent with this conclusion, another study recently reported resensitization to tamoxifen by silencing the SRC3.[91] Additionally, in cells that overexpress HER-2, the agonistic activity of tamoxifen was reverted to an antagonist action by using inhibitors of HER-2
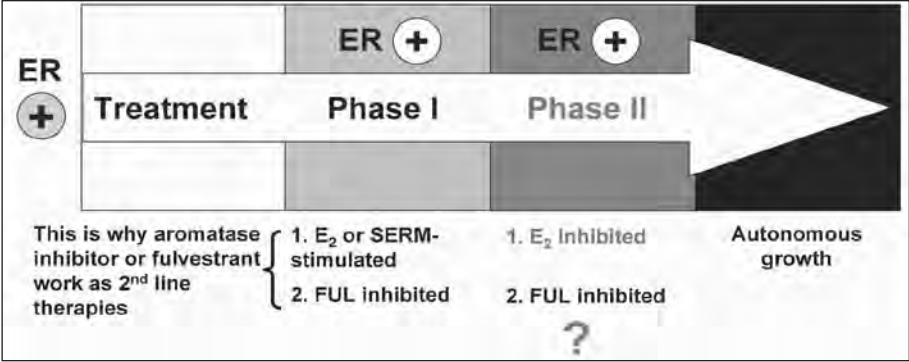
Figure 2. Diagram depicting different phases of SERM resistant breast cancers.

signaling.[82,90] This being the case, it is therefore important to understand the underlying mechanism of HER-2 initiated signaling cascades so that new therapeutic strategies can be formulated. Phosphatidylinositol-3-kinase (PI3K)/AKT and mitogen-activated protein kinases (MAPK) are the two critical signaling pathways which are activated aberrantly, in cells that overexpress HER-2.[92] Indeed, activation of AKT in ER positive breast cancer patients predicts decreased overall survival in tamoxifen treated patients.[93,94] Estrogen can rapidly activate AKT via the HER-2 pathway in cells expressing low levels of HER-2 and 4-hydroxytamoxifen can block this activation.[95] However, in breast cancer cells overexpressing HER-2, 4-hydroxytamoxifen can also activate AKT pathway in
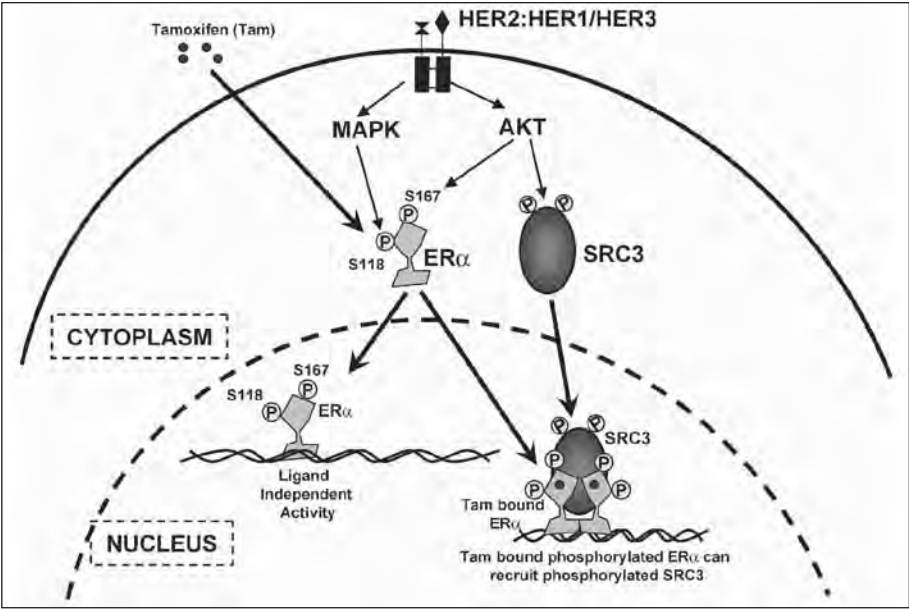


Figure 3. Schematic representation of cross talk between HER2 and estrogen signaling pathways. High HER2 expression activates AKT and MAPK pathways which can phosphorylate estrogen receptor (ER) and steroid coactivator 3 (SRC3). Phosphorylated ER can activate transcription independent of ligand. Tamoxifen bound phosphorylated ER can recruit phosphorylated SRC3 instead of corepressors and act as an estrogen agonist.

a HER-2 dependent manner,[90] exemplifying the conversion of 4-hydroxytamoxifen to an agonist. Both AKT and MAPK pathways can phosphorylate ER as well as the coactivator AIB1 (SRC3). Serine 167 residue of ER can be phosphorylated by AKT,[96] whereas serine 118 residue of ER can be phosphorylated by the MAPK pathway, both resulting in ligand-independent activation of estrogen receptor.[97,98] Not surprisingly, breast cancers with high levels of SRC3 along with HER-2 over-expression are associated with worse outcome following tamoxifen therapy, indicating resistance.[99] A recent study have also reported that specific phosphorylation of ER can modify the binding ability of ligands and also modulate its capacity to interact with co-activators.[100] In addition to HER-2, elevated level of EGFR/HER-1, another member of the EGFR family, is also correlated with poor prognosis and has been implicated in SERM resistant breast cancers.[101,102] Different members of EGFR family can dimerize, autophosphorylate and activate different signaling pathways. Long term treatment with tamoxifen, resulting in resistance, is also associated with increased translocation of ER α out of the nucleus and enhanced interaction with EGFR.[103] Similarly, high levels of HER-2 were found to increase the relocalization of ER α from nucleus to cytoplasm.[104] It is therefore evident from these findings that aberrant signaling cascades initiated by over-expressing EGFR and HER-2, particularly involving PI3K/AKT and MAPK pathways, are critically involved in cross talk with the genomic components of ER responses. All of these events may merge to create resistance to SERM treatment.

### *Other Factors Involved in SERM Resistant Breast Cancers*

In addition to aberrant activation of AKT and MAPK pathways in SERM resistant breast cancers, several other factors have also been reported. The mammalian target of rapamycin (mTOR), which is a downstream target of PI3K/AKT and MAPK pathway,[105,106] is found to be involved in estrogen induced proliferation of ER positive breast cancer cells.[107,108] Furthermore, specific inhibitors of the mTOR pathway restore sensitivity to tamoxifen in a tamoxifen resistant cell line, both in vitro and in vivo.[109]

Another downstream target of EGFR and HER-2, is c-Src which phosphorylates p27 and impairs its inhibitory action on cyclin dependent kinase 2 (Cdk2) resulting in increased mitogenic activity. This mechanism is also implicated in tamoxifen resistance, as inhibition of c-Src was found to restore tamoxifen sensitivity.[110]

A rather novel approach to reversing tamoxifen resistance is to use disulfide benzamide (DIBA) that disrupts the zinc fingers of ER DNA binding domain and prevents the association of coactivators with 4-hydroxytamoxifen bound ER. DIBA was able to restore the tamoxifen sensitivity in several different tamoxifen resistant cells. However, this effect was achieved without altering the phosphorylation statuses of HER-2, MAPK, AKT and AIB1 in these cells.[111] It is possible that the use of DIBA with an inhibitor of phosphorylation would be a reasonable strategy for long term therapeutic use.

### Therapeutic Options for SERM Resistant Breast Cancers

Since EGFR and HER-2 mediated signaling events play important roles in SERM resistant phenotype of breast cancers, blocking these pathways represent a logical approach in combating SERM resistance. Indeed, several laboratory studies have used selective inhibitors of HER-2 and/or EGFR in SERM resistant cells and reported beneficial outcomes, including reversal of SERM resistance.[90] A recent study[112] demonstrates that using a combination of three drugs, all targeting the HER2 by different mechanisms, along with tamoxifen or estrogen deprivation could effectively block the growth of HER2 overexpressing ER positive breast cancer in athymic mice. In another study using raloxifene resistant breast cancer cells, blocking of HER-2 activation by trastuzumab (humanized monoclonal antibody against HER-2) was found to decrease the growth of the resistant tumors in laboratory animals.[85] This approach was particularly effective in preventing the growth of tamoxifen stimulated endometrial cancers.[113] Clinical efforts are therefore directed towards using either small molecule inhibitors against EGFR and HER-2 or humanized monoclonal antibody against HER-2 as a monotherapy or in combination with other therapies

including SERMs, in patients not responding to endocrine therapies.[114] As mentioned earlier aromatase inhibitors or fulvestrant are equally effective at treating breast cancer patients who are already resistant to tamoxifen. However, laboratory studies[115] now show that the initial inhibition of tumor growth, by either fulvestrant or estrogen deprivation is quickly followed by resistance and all the resistant tumors exhibit elevated levels of phosphorylated AKT and MAPK.[115] Tumor control by fulvestrant or estrogen deprivation is enhanced when this approach is combined with therapy that inhibits the EGFR/HER-2 signaling. These findings further underscore the idea that inhibiting the downstream targets of AKT and MAPK pathway, like mTOR, may be of significant importance in attenuation of SERM resistance.[109]

## Resistance to Long Term Antihormone Therapy

The laboratory models and mechanisms discussed so far really represent the early stages of drug resistance to SERMs. The models replicate treatment of metastatic breast cancer with tamoxifen and do not replicate the strategy of long term adjuvant therapy with 5 years of tamoxifen. To address this deficiency tamoxifen-stimulated breast tumors have been repeatedly transplanted into tamoxifen-treated athymic mice to replicate micrometastases that grow in a tamoxifen environment for years. Remarkably, the signal transduction pathways in tumor cells become reconfigured so that estrogen is no longer a survival signal but triggers apoptosis in phase II resistant breast cancer cells[116-118] (Fig. 2).

## Estrogen Induced Apoptosis

Phase II tamoxifen stimulated tumors are dependent upon tamoxifen for growth and are cross resistant with raloxifene.[119] Indeed the converse is also true. Raloxifene-resistant breast cancer cells can be grown into tumors in athymic mice by treatment with either raloxifene or tamoxifen.[118] However, it is the dramatic antitumor effect of estrogen as a major factor in breast tumor cell survival that is intriguing. High dose estrogen therapy was originally used as a palliative treatment for postmenopausal metastatic breast cancer before tamoxifen, an antiestrogen, was developed during the 1970's.[11] Alexander Haddow[120] reported that high doses of synthetic estrogens would produce a 30% response rate in unselected patients and the responses would last about one year. Despite the fact that treatment with high dose estrogen therapy has slipped into disuse with the ubiquitous use of tamoxifen and new aromatase inhibitors, recent laboratory studies indicate that low dose, rather than high dose, estrogen could again find a place in the treatment paradigm of metastatic breast cancer. The first indication that this was true occurred when the findings that physiologic level of circulating estradiol could cause tumor regression in long term tamoxifen resistant tumors (phase II).[116,117] The idea is now being advanced to the clinic as there is every reason to believe that the concept will translate as a treatment for antihormone resistant breast cancer. It is already known that high dose estrogen produces a 30% response rate in patients whose tumors are refractory following exhaustive antihormonal therapy.[121]

Additionally the paradoxical effect of estrogen to induce apoptosis is not limited to SERM resistant breast cancer cells, but has also been observed in estrogen deprived breast cancer cells.[122,123] Although the precise mechanism of estrogen induced apoptosis is under intense investigation, studies have indicated the involvement of mitochondrial pathway of apoptosis in estrogen deprived cells,[124] and a different mechanism in raloxifene resistant cells.[118] Most importantly, laboratory studies have shown that the breast cancer cells that become resistant to estrogen induced apoptosis regain the sensitivity for SERM therapy.[117] Therefore, it is possible that cyclical treatments with SERM and estrogen may help to control breast cancer growth for a prolonged period.[125]

## Conclusion

Currently, tamoxifen, the prototypical SERM, can be used to treat all stages of ER positive breast cancers and for chemoprevention in high risk women. The effectiveness of this class of drugs is based on selectively blocking the estrogen mediated effects in the breast cancer. The fact that the ER is such an important target and that majority of breast tumors are ER positive has made ER

blockade such a significant therapeutic success. This clinical success has led to the development of other SERMs in the group, like raloxifene, with fewer undesirable effects. However, despite significant advances the use of long term SERM treatment is ultimately associated with acquired breast cancer resistance. Nevertheless, studies during the past decade have identified specific signaling pathways that are involved in the cross talk with ER signaling, thereby creating resistance to SERMs. Although encouraging results and strategies are being developed to employ inhibitors of phosphorylation pathways it may be that the tumors develop too many signaling options to use a single approach to block resistance. In this regard the novel finding that estrogen will eventually induce apoptosis in SERM resistant breast cancer cells merits further detailed study for its wider therapeutic use. It may be that the skill of the ER to activate apoptosis can be used to identify an apoptotic trigger to kill cancer cells selectively.

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# Low Dose Estrogen Therapy to Reverse Acquired

# Antihormonal Resistance in the Treatment of Breast Cancer

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**Abstract**

Estrogen is a potent stimulus for growth in its target organs; the uterus, vagina and some estrogen receptor positive breast cancers. However, estrogen is also able to control menopausal symptoms and maintain bone density in postmenopausal women. Until recently, there was also believed to be a link between estrogen and the prevention of cardiovascular disease. For these reasons, hormone replacement therapy (HRT) with an orally active estrogen and progestin has been used routinely for more than 50 years to maintain physiologic homeostasis after menopause. Not surprisingly, HRT increases the risk of developing breast cancer. The link between estrogen and breast cancer growth served as the incentive to develop long term tamoxifen therapy and subsequently the aromatase inhibitors, as successful "antiestrogenic" treatments. Unfortunately, the consequence of exhaustive therapy is drug resistance. Laboratory studies have defined the evolution of tumor drug resistance to tamoxifen, raloxifene (used for breast and osteoporosis chemoprevention), and the aromatase inhibitors. Remarkably, the long term exposure of breast cancers to antihormonal therapy also exposes a vulnerability that is being exploited in the clinic. Years of antihormonal therapy alters the cellular response mechanism to estrogen. Normally, estrogen is classified as a survival signal in breast cancer but in sensitive antihormone resistant cells, estrogen induces apoptosis. Once resistant cells are killed, antihormonal therapy is once again effective. This new targeted approach to the treatment of metastatic breast cancer could open the door to novel approaches to treatment with drug combinations.

**Introduction**

Estrogen is essential for life. Without estrogen there would be no human race; reproduction would be impossible. However, with the evolution of the human race and the development of functional societies has come the promise of an extended life through the control and in some cases, conquest of disease.

The end of the 19th century was a period of important medical advances with the introduction of vaccines and the start of the chemotherapeutic era for infectious diseases. Life expectancy for women was short – 44.46 years.[1] After a century of implementing public health advances with vaccination and antibacterial therapies, life expectancy for women is now 80.8 years.[2] This is true for all developed countries, but with success in public health comes new challenges for a population that is larger than ever before.

Cancer is essentially a disease of advancing years. Specifically, breast cancer is rare in women under 30 years of age (4 per 100,000 women), but increases dramatically during the next 40 years of life. The incidence of breast cancer in a population of 70-75 year old women is 400 per 100,000 women per year. Although there is some emerging evidence that estrogen can cause transformation of breast or mammary cells,[3] there is evidence from prospective studies that the practice of prescribing hormone replacement therapy (HRT) to prevent osteoporosis and hypothetically to prevent aging has significantly increased breast cancer incidence.[4-6] A brief examination of why HRT became so fashionable and the current clinical concerns will serve as a physiological background to address the rationale for the development of endocrine therapies (high dose) sex hormones or antihormones for breast cancer treatment over the past 50 years.

**Hormone replacement therapy**

The initial goal for estrogen replacement was to ameliorate the menopausal symptoms that occurred once ovarian estrogen synthesis ceased. Subsequently, the focus was to maintain bone density or prevent increases in coronary heart disease in women during later life. Two approaches occurred to enhance and maintain the physiologic actions of estrogen past the menopause.

Synthetic estrogens based either on the structure of triphenylethylene or the very potent, but shorter acting diethylstilbestrol [7, 8] (Figure 1), were described in the literature and they proved to be a cheap source of new medicines. High dose synthetic estrogen administration was found to be effective in the treatment of breast and prostate cancer, [9] but even low doses of synthetic estrogens never really became accepted as HRT in postmenopausal women. Indeed, diethylstilbestrol subsequently achieved notoriety as an estrogen supplement to prevent recurrent abortion. Children of treated mothers had a high incidence of clear cell carcinoma of the vagina[10, 11]. In contrast, the synthetic estrogens based on triphenylethylenes were subsequently to undergo a metamorphosis and be transformed into antiestrogens used for the treatment of breast cancer[12] (Figure 2).

The estrogen (Figure 3) derived from pregnant mares (Premarin®) was initially used as an estrogen replacement therapy for postmenopausal women. However it found that there was a 6 fold elevation in endometrial cancer.[13, 14] The stimulatory action of estrogen in the uterus was neutralized by combining the orally active estrogen with the synthetic progestin medroxyprogesterone acetate (MPA) as Prem Pro®. This preparation was used by patients for up to a decade to prevent osteoporosis, menopausal symptoms,

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and was also taken by many women in the belief it would prevent aging and coronary heart disease (CHD).

The actual link between HRT and breast cancer was addressed prospectively in two studies initiated during the 1990's. The Women's Health Initiative (WHI) recruited 16,608 women between the ages of 50-79 years who received either conjugated equine estrogen 0.625mg/day plus MPA 2.5 mg/d or placebo. The primary outcome was CHD with invasive breast cancer as the primary adverse outcome. The Million Women Study recruited 1,084,110 women (aged 50-64 years) to determine the effects of specific types of HRT on incidence and fatal breast cancer.

The WHI, with a mean follow up of 5.2 years, was stopped prematurely because invasive breast cancer incidence exceeded the stopping boundary. [4] Overall, it was found that breast cancers were diagnosed in the HRT treated women at a later stage compared to placebo, possibly because there was an increase in mammographic density.[6] Overall, the study investigators did not find that HRT should be used to reduce the risk of CHD.[4] However, a recent sub-analysis of younger women in the group indicates minor benefit.[15]

The Million Women's Study[5] concluded that HRT is associated with an increased risk of incidence of fatal breast cancer, particularly if the HRT was an estrogen/progestin combination. The authors estimated that, over the decade 1993-2003, HRT had increased the incidence of invasive breast cancer in the United Kingdom by an excess of 20,000 new breast cancers.

It is interesting to note that with the publication of both the WHI Study and the Million Women's Study in the first 5 years of the 21st Century, there has been a

significant decline in the prescribing of HRT.[16-20] As a result, this has been associated with a drop in the incidence of breast cancer.[21]

Thus, estrogen has a justified reputation as a potent stimulant of breast cancer development and growth. This reputation led to the development of antiestrogenic targeted strategies to treat and prevent breast cancer.

**Antiestrogenic treatment strategies**

In the latter part of the 19th Century, farmers in Scotland ovariectomized their farm animals to extend milk production. The observation had also been made that the histology of the lactating breast was similar to breast cancer. This knowledge was subsequently used by George Beaston[22] to justify the oophorectomy of a young woman who had inoperable advanced metastatic disease breast cancer. The woman responded dramatically but further evaluation of the concept demonstrated that only one in three women would have effective disease control for about 1-3 years.[23] Nevertheless, the concept of endocrine ablation as a standard treatment for metastatic breast cancer was subsequently extended to postmenopausal women with the use of adrenalectomy and hypophysectomy.[24] The response rate remained at 30% but it was not until the pioneering work of Elwood Jensen[25] and the identification of the estrogen receptor (ER) that progress was made in understanding estrogen regulated growth mechanisms. The development of the ER assay that was primarily used to exclude those women who would *not* respond to endocrine ablation, was an important step forward in breast cancer treatment.[26, 27] Looked at in another way, the presence of the ER in a breast tumor increased the probability that endocrine ablation would be successful. Since this was the era before tamoxifen, it also suggested a use for a drug ICI 46,474, discovered in the

antifertility program at Imperial Chemical Industries (ICI) Pharmaceuticals Division (now AstraZeneca). The compound failed in its primary application as an antifertility agent[28] because, like clomiphene,[29] it induced ovulation in subfertile women.[30] The compound was found to have modest activity as a treatment for unselected breast cancer[31] but ICI 46,474 was subsequently reinvented[32] during the 1970's as a targeted therapy for breast cancer. A scientific foundation was established in the laboratory for the treatment and prevention of breast cancer[33-35] by blocking estrogen action at the level of the ER.[36]

Coincidentally, another approach to controlling the growth of estrogen stimulated breast cancer was also emerging in the 1970's with the specific targeting of the aromatase enzyme CYP19 that converts androstenedione or testosterone into estrone or estradiol respectively in postmenopausal patients.[37] The first clinically useful specific aromatase inhibitor was 4-hydroxyandrostenedione that binds irreversibly to the active site of the enzyme.[38] There are now numerous aromatase inhibitors that bind either irreversibly or competitively at the active site of the aromatase enzyme.

**Transition to Tamoxifen.**

Prior to 1981, the standard of care for the palliative treatment of post-menopausal women with metastatic breast cancer included high dose estrogen treatment.[24] Although the mechanism of action was unknown, treatment with diethylstilbestrol (DES) was accepted as being among the most effective of the medical hormonal manipulations employed with expected response rates (RR) of approximately 36%. Other common hormonal approaches included "androgenization" with androgens (21% RR), high dose progestins, used either as a single agent or in combination with estrogen, and the use of

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glucocorticosteroids as a means of chemical adrenalectomy to interrupt the hormonal feedback-stimulation axis. These additional hormonal therapies resulted in expected RR ranging from 15 to 50%, with the lower figures being more realistic.

ICI 46,474, also known as tamoxifen, is a non-steroidal antiestrogen demonstrated in animal laboratory models to oppose the action of estrogens.[39] An early clinical appraisal of this agent was initially undertaken in 46 post-menopausal patients with metastatic breast cancer whose treatment had progressed after prior treatment with hormonal therapies.[31] Of the 46 patients treated with tamoxifen for at least 3 months, 10 patients (21%) demonstrated partial or complete response. Additionally, 17 patients (37%) experienced stable disease with some experiencing response of visceral metastases as well. Tamoxifen was well tolerated with few serious side effects. Hot flushes and nausea and vomiting were the most significant side effects resulting in treatment discontinuation in a few (4%).

Based on this and other encouraging data,[40] a randomized clinical evaluation of tamoxifen and diethylstilbestrol was undertaken.[41] One hundred and fifty-one post-menopausal women with metastatic breast cancer and measurable disease who may have been previously treated with chemotherapy, but had not been treated with previous hormonal therapies for metastatic disease, were randomized to treatment with either tamoxifen (10 mg 2 x daily) or diethylstilbestrol (5 mg 3 x daily). Treatment with diethylstilbestrol (RR = 41%) resulted in higher response rates (RR) than tamoxifen (RR = 33%), but the difference was not statistically significant. Clinical benefit rates (Clinical benefit = complete response + partial response + stable disease) of 84% and 78% were also similar for both tamoxifen and diethylstilbestrol, respectively. Toxicity profiles

favored tamoxifen with significantly lower rates of nausea and vomiting, edema, and vaginal bleeding. Several smaller randomized trials also confirmed these findings.[42-44] No significant differences between estrogen preparations and tamoxifen with respect to reported response rates (ranging from 25% to 53%), rates of clinical benefit, and/or duration of response were found. Because tamoxifen was associated with fewer side effects without loss of efficacy, it replaced DES as the first-line medical intervention of choice for post-menopausal women with metastatic breast cancer. Updated long-term follow-up analysis of greater than 14 years have confirmed the initial reported response rates.[45] However, of interest, with longer follow-up, 5-year survival is significantly superior (adjusted p = 0.039) for the patients treated with DES (35%) compared to those treated with tamoxifen (16%).

Tamoxifen dosing was modeled in the laboratory to show that early chronic dosing of rats was more important at preventing mammary cancer development than larger interval doses.[28, 46, 47] These translational animal studies *in vivo* established the current standard use of long-term adjuvant antiestrogen therapy chronically administered to prevent breast cancer recurrence. Five years of adjuvant tamoxifen is known to reduce both the local recurrence as well as distant metastatic disease by approximately 50% in patients whose breast cancer expresses the estrogen receptor (ER).[48] Adjuvant tamoxifen also reduces the risk of breast cancer mortality by approximately one-third.[48]

**Long term antihormonal therapy**

The scientific strategy[36] of targeting those breast tumors with the ER with long-term antihormonal therapy[47] has now reached its zenith. Long-term antihormonal adjuvant therapy is routine for patients with an ER positive tumor and several clinical

facts are now clear. Five years of adjuvant tamoxifen therapy is now considered sufficient to provide long-term survival benefits for patients[49] and the antitumor effects of tamoxifen extend for at least 10 years following a five year course of adjuvant therapy.[48] Side effects in postmenopausal women using tamoxifen are principally increases in endometrial cancer risk and blood clots. Although the risk benefit ratio is acceptable when tamoxifen is used as a therapy, this is not acceptable for postmenopausal women wishing to reduce the risk of breast cancer.[50-52] Aromatase inhibitors used for breast cancer treatment improve both survival and reduce concerns about blood clots and endometrial cancer,[53-56] but there is a potential concern about osteoporosis that can be adequately addressed with bisphosphonate treatment for women with either osteopenia or osteoporosis. No results are as yet available for the use of aromatase inhibitors as chemopreventive agents but the SERM raloxifene is available for the prevention of osteoporosis with, as predicted,[57-60] the prevention of breast cancer as a beneficial side effect.[61, 62] The use of raloxifene for this indication by one-half million osteoporotic women reduce breast cancer incidence by approximately 27,000 over ten years.[63] Recently, the application of raloxifene has been extended to primary chemoprevention in high risk postmenopausal women.[64]

Each of the applications of SERMs or aromatase inhibitors described above uses a 5 year treatment period. A small study demonstrated that longer term tamoxifen extending to 10 years did not improve recurrence rates but did increase accumulated side effects.[65] In contrast, the application of a non cross resistant aromatase inhibitor following 5 years of tamoxifen improves not only disease-free survival, but reduces the incidence of side effects and contralateral breast cancer.[66, 67] Thus, the proposal[47] of

using a SERM followed by estrogen deprivation has now become a clinical realty and long-term antihormonal therapy for the treatment and prevention of breast cancer is the standard of care. However, the ubiquitous application of antihormones in medicines now has consequences for breast cancer cells potentially exposed to estrogen deprivation for a decade. The treatment of antihormonal drug resistance is a challenge that needs to be addressed to develop cheap and effective future interventions.

**Drug Resistance to Tamoxifen – Evolution from Benefit to Liability**.

With the advent of newer third generation selective aromatase inhibitors, it is common practice for post-menopausal patients to be treated with tamoxifen followed by extended adjuvant antiestrogen therapy with an aromatase inhibitor, resulting in at least 5-10 years total of chronic, continuous antiestrogen blockade [66]. However, antiestrogen therapy is not able to prevent all recurrences, suggesting that despite the presence of the ER, a majority of tumors become resistant. In fact, continuous extended therapy tamoxifen has consequences for initially estrogen responsive breast cancer cells. Here again, pre-clinical *in vivo* modeling has provided a scientific insight. The estrogen responsive ER positive breast cancer cell line MCF-7[68] has been successfully grown into tumors by inoculation into athymic mice. Subsequent treatment with long-term tamoxifen has been used to mimic the effects of adjuvant therapy. Years of treatment are replicated by serially transplanting any growing tumors into tamoxifen treated athymic, ovariectomized mice. Initially, tumors established in the presence of estrogen are growth suppressed by tamoxifen, maintaining cytostatic activity without progressive increase in size[69] for several months. However, eventually tamoxifen stimulated tumors start to grow but the tumors also grow in response to physiological estradiol levels.[69] These

characteristics are described as Phase I selective estrogen receptor modulator (SERM) resistance where either a SERM (e.g. – tamoxifen or raloxifene[70]) or estrogen can stimulate tumor growth in cells previously exposed to treatment with long term tamoxifen or SERM therapy (Figure 4). In the clinic, Phase I tumor resistance is usually treated with either an aromatase inhibitor or fulvestrant to destroy the ER.[71, 72]

**A new biology of estrogen action**

If long-term tamoxifen treated tumors continue to be passaged for 4-5 years to mimic adjuvant tamoxifen therapy, they acquire molecular changes associated with an unanticipated vulnerability. Selective ER modulator stimulated growth is thought to be mediated by anti-apoptotic pathways.[73, 74] Unexpectedly, estrogen, rather than promoting growth of these long-term estrogen-deprived cells, now produces a tumoricidal effect.[75, 76] To confirm this laboratory finding, fresh mice were "bi-transplanted" with both newly established MCF-7 tumor as well as long term tamoxifen resistant MCF-7 tumor within the same animal on different sides of the axillary region of the mammary fat pads. When treated with tamoxifen, the wild MCF-7 tumor did not grow in response to tamoxifen treatment, while the tamoxifen resistant MCF-7 tumor grew. In contrast, estrogen stimulated the wild type MCF-7 tumor to grow but the long term tamoxifen resistance tumor did not grow. This suggested that the difference in response was not due to a difference in the host having an enhanced or altered response to estrogens and/or tamoxifen, but rather a property inherent to the ER positive breast cancer cells acquired in the setting of chronic estrogen deprivation over long periods of time.[76] These characteristics are described as Phase II of SERM resistance where ER positive tumors are stimulated to grow by tamoxifen, but killed by estrogen.

There is also another consequence of Phase II SERM resistance; Laboratory studies. Fulvestrant, the pure antiestrogen, is able to prevent Phase II tumor growth after tamoxifen withdrawal and the results are comparable to no treatament.[74]  Again, these laboratory results are consistent with the clinical use of fulvestrant or an aromatase inhibitor following the development of tamoxifen resistance.[71, 72]  However, the laboratory finding that physiological estrogen plus fulvestrant causes robust tumor growth[74, 77] raises the question of a negative drug interaction between fulvestrant and physiologic estradiol.  The inhibitor actions of each agent are cancelled out by the combination.  Fulvestrant is not very active as a third line agent which raises the possibility that the estrogen already present in the postmenopausal woman may interfere in an unanticipated fashion with the inhibitory action of the pure antiestrogen.  Clinical studies are ongoing, examining the efficacy of a fulvestrant/aromatase inhibitor combination.

Overall, the recognition of the new biology of estrogen action observed following the development of long-term tamoxifen treatment raises the question of the global relevance of the observation to estrogen withdrawal following treatment with aromatase inhibitors and the potential exploitation of the new knowledge of mechanisms can be identified.

**Long-term Estrogen Withdrawal Apoptotic Mechanisms.**

The increasing clinical use of aromatase inhibitors to reduce estrogen synthesis as a strategy to treat breast cancer has resulted in increased efforts to examine drug resistance to estrogen withdrawal rather than SERM action.  Early studies growing MCF-7 breast cancer cells in estrogen free media resulted in an increase in intracellular ER

levels and spontaneous cell growth.[78, 79]  Several estrogen independent clones were isolated for study[80, 81] and ideas were proposed that MCF-7 cells are hypersensitized to grow in extremely low levels of estrogen: i.e. below the level that can be detected or further reduced.[82]  However, Song and coworkers[83] observed that increasing concentrations of estradiol could increase apoptosis in estrogen deprived cells by increasing the concentration of FASL that activates death receptor pathways.  Thus, the original observations that Phase II tamoxifen resistant tumors could be treated with physiologic estrogen[75, 76] were extended to aromatase inhibitor resistant cells. However, in contrast to Song's study[83], Phase II tamoxifen resistant tumors respond to increasing estrogen treatment by increasing the FAS receptor, decreasing HER2/neu, and NFκB that is associated with tumor regression.[74]  Furthermore, MCF-7 cells kept for many years under estrogen directed conditions using medium containing stripped fetal bovine serum produce rapid apoptosis via an intrinsic medium diverted at the mitochondrion.[84, 85] However, both Lewis[85] and Song[86] find that apoptosis is modulated through bcl-2 or bcl-2XL.  A representative schema based on the studies of Lewis and coworkers[85] as shown in Figure 5.

It is also perhaps important to state that the new knowledge is emerging through re-examination of existing cell lines.  In early publications studying the effects of estrogen withdrawal, no estrogen-induced apoptosis was noted[80, 81] but by altering culture conditions or extending the period of estrogen exposure, apoptosis occurs.[84, 87]  Overall, the phenomenon observed with long-term estrogen withdrawal is similar to the Phase II resistance of the model described for SERMs.

**Clinical Clues.**

In the clinic, patients with ER positive breast cancer are treated with exhaustive antiestrogen therapies. However, over time and with sequential antiestrogen therapy, antiestrogen resistance can be expected to occur in as many as 50%.[88] With each successive antiestrogen treatment of such recurrent tumors, tumor response becomes less durable. Also, the combination of tamoxifen plus DES was no better than tamoxifen alone.[88] Lonning and coworkers[89] addressed the hypothesis that patients with ER positive breast cancers who had been treated exhaustively with antihormonal therapy could potentially respond to high dose estrogen therapy. Thirty-two patients with advanced breast cancer previously exposed to between 2 and 10 (median 4) endocrine treatments were treated with DES (5 mg three times daily). Therapy was well tolerated but 4 patients terminated treatment within 2 weeks of starting and another two stopped treatment before progress. One of these patients had stable disease for 15 weeks and one a partial response for 39 weeks. Of the remainder, four patients obtained a complete response and six patients, a partial response. Two patients had stable disease for six months and one $\geq$ one year. Overall, these extremely encouraging preliminary studies with high dose estrogen therapy are complimented by anecdotal reports of the effectiveness of low dose estrogen treatment for those women with endocrine refractory breast cancer following exhaustive antihormonal therapy (Dr. James Ingle, Mr. Michael Dixon, personal communications). As a result, several clinical studies are currently underway (Drs. Matthew Ellis and Richard Santen, personal communications).

**Estrogen-induced apoptosis:  clinical and laboratory correlations**

Based on the pre-clinical laboratory modeling, we have translated the new biology of estrogen action into a Department of Defense Center of Excellence grant with laboratory and clinical collaborators illustrated in Figure 6.  Our goal is to define the pathways for estrogen induced survival and apoptosis in endocrine responsive breast and endometrial cancer and use the emerging database to guide the interpretation and development of a series of clinical trials.  The ultimate goal of our clinical trial design is illustrated in Figure 7 and currently consists of two separate but interconnected therapeutic estrogen trials.

In Trial I, "A Single Arm Phase II Study of Pharmacologic Dose Estrogen in Postmenopausal Women with Hormone Receptor-Positive Metastatic Breast Cancer After Failure of Sequential Endocrine Therapies" eighty eight patients who have clearly responded and failed at least two antiestrogenic therapies will be treated for 12 weeks with 30mg estradiol (Estrace®).  Patients who respond or have stable disease will be treated subsequently with 1 mg anastrozole until disease progression.  Serum and, where possible, recurrent tissue biopsies will be used to determine serum apoptotic markers (Apoptosense®) and target genes in tumor material as markers of apoptosis or tumor progression.  These data will be compared and contrasted with the results obtain from preclinical studies using our cell and animal models.

In Trial 2 **"**Reversal of Anti-Estrogen Resistance with Sequential Dose De-escalation of Pharmacologic Estrogen in a Single Arm Phase II Study of Postmenopausal Women with Hormone Receptor-Positive Metastatic Breast Cancer After Failure of Sequential Endocrine Therapies", patients who have responded and subsequently failed two

antiestrogenic therapies will be treated as groups with successively lower doses of daily estradiol to determine the lowest dose necessary to produce an equivalent response to 30 mg estradiol in Trial 1.

On completion of the integrated research program, several questions can be addressed to improve treatment of metastatic breast cancer:

1) Can a select group of patients be identified from either tumor markers or early serum apoptotic products who will respond to limited low dose estradiol treatment and who will subsequently remain under disease control with anastrozole treatment?

2) Can cell survival pathways be identified for tumors that do not respond to estradiol treatment?

3) Can survival pathways be subverted to improve response rates to estradiol-induced apoptosis?

**Conclusions**

The development and extensive clinical application of long-term antihormonal therapy[37] has had consequences for the patient with the development of antihormonal drug resistance in some breast cancers.[90] However, with the development of drug resistance to exhaustive antihormonal therapy, a vulnerability of the cancer has been exposed. The recognition of the new biology of estrogen action that causes apoptosis in sensitive breast tumors now opens an unanticipated door of opportunity to exploit the findings to aid patients. Although the actual clinical responses may not be profound in unselected patient populations or in populations whose tumors do not have the correct (Stage II) form of breast cancer, our ability to decipher apoptotic mechanisms from

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laboratory models and eventually target patients appropriately, may eventually have profound positive effects for some patients. The translational knowledge gained over the next few years may again provide unanticipated opportunities to exploit the discovery of "apoptotic triggers" for other forms of cancer.

It is perhaps pertinent to restate that for 70 years there has been an "ebb and flow" relationship in the role of estrogen in breast tumor homeostasis. We have illustrated in this review many of the changing fashions that have occurred in how estrogen is perceived as a benefit or a villain in women's health. The effects of modulating the ER system in the breast, at one time or another, have been dismissed because they are small or believed to be of no major consequence. Nevertheless, the small observations become accumulative. By way of example, it is important to recall that initial use of tamoxifen, a failed contraceptive, to treat unselected populations showed only modest responses for some patients with metastatic breast cancer.[32] Years later, after deciphering the target populations and translating the appropriate treatment strategies from the laboratory to the clinic, the drug became the gold standard for endocrine therapy[32] and was credited with improving the survival of hundreds of thousands of women.[48] The challenge for the future is to exploit the profound apoptotic action of estradiol as a lead to develop innovative new therapies for cancer.

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**Figure Legends**

**Figure 1.** The evolution in structure function relationships of estrogens based on diethylstilbestrol. This potent estrogen has a high affinity for the estrogen receptor (ER) and historically was used, at high doses (15 mg. daily) to treat both breast and prostate cancer. Description of the metabolic activation of tamoxifen to 4-hydroxytamoxifen[91, 92] was the first clue that tamoxifen was a prodrug and needed to be converted to metabolites with a high binding affinity for ER. Raloxifene (formerly the failed breast cancer drug keoxifene[93]) used knowledge from prior structure function studies to design an antiestrogen with low uterotropic action but a high affinity for ER. The compound is a selective ER modulator (SERM) used for the long term treatment and prevention of osteoporosis and the prevention of breast cancer.[61, 64] Raloxifene, unlike tamoxifen, has not been found to increase uterine hyperplasia or increase the incidence of endometrial cancer.[64, 94]

**Figure 2.** The evolution in structure function relationships of antiestrogens based on triphenylchlorethylene.[8] The long acting estrogen triphenylchlorethylene was used as a treatment for breast cancer[9] and served as the basis for the discovery of clomiphene (a mixture of estrogenic and antiestrogenic *cis* and *trans* isomers [95] used for the treatment of infertility.[29] Clomiphene was not used to treat breast cancer because of concerns about toxicity but served as the lead compound for the subsequent synthesis of tamoxifen, the pure trans isomer of a triphenylethylene,[28] that was eventually developed for the targeted treatment and prevention of breast cancer.[32]

**Figure 3.** The natural estrogens 17β estradiol and estrone are metabolically interconverted in women. The orally active estrogen preparation Premarin® is obtained by extracting pregnant mare's urine. The principal estrogen is estrone sulphate which can be activated to estrone with sulphatase. Estrone in turn can then be converted by 17 hydroxy steroid dehydrogenase to the potent estrogen 17β estradiol. The other minor compounds in Premarin® are equilin and equilenin. Both are weak estrogens.

**Figure 4.** The evolution of antihormonal resistance in breast cancer. A. The current clinical view of drug resistance to tamoxifen or any selective estrogen receptor modulator (SERM). Long term tamoxifen treatment eventually selects for tamoxifen stimulated tumor growth. These tumors, are recognized by responding to tamoxifen withdrawal [96] but also grow in response to physiologic correlation of estrogen. These observations are supported by laboratory studies [69]. This form of tamoxifen resistance forms the basis for the response of patients to either aromatase inhibitors or fulvestrant following tamoxifen failure [71, 72] and the basis of the success of extended antihormonal therapy with five years of tamoxifen followed by five years of an aromatase inhibitor [66]. B. The emerging laboratory view of drug resistance to SERM or aromatase inhibitors. Drug resistance evolves to a point where the tumor is exclusively dependent on the SERM (Tamoxifen and raloxifene) or there is autonomous growth via the ER with long term estrogen withdrawal. The biology of estrogen changes dramatically as the tumor cell evolves from Phase I to Phase II. Estrogen now becomes an inhibitory or apoptotic signal. These emerging new laboratory data have important implications for future clinical practice.

**Figure 5.** A summary of sequence of subcellular events that occur in experimental models during estradiol-induced apoptosis in breast cancer. In some models, estradiol increases Fas ligand(L)[83] but in others, Fas receptor increases and there is a reduction in the survival signals from Her2/neu and NFκB[74]. In contrast, Lewis and coworkers[85] have described the actions of estradiol mediated through a mitochondrial mechanism.


**Figure 6.** The organization of our Department of Defense Center of Excellence Grant entitled "A New Therapeutic Paradigm for Breast Cancer Exploiting Low-Dose Estrogen-Induced Apoptosis." The model systems to study the survival and apoptosis induced with estrogen are being used for time course experiments at the Fox Chase Cancer Center. The materials are distributed to Translational Genomics for genomic analysis using CGH, siRNA analysis or agilent gene array analysis, and the Vincent T. Lombardi Cancer Center is involved to conduct proteomics. All results are uploaded into a shared secure web for data processing and target identification by our informatics and biostatistical group. Each laboratory is able to validate emerging pathways and study individual genes of interest. Our program is integrated with a clinical trials program that provides patient samples for validation of apoptotic or survival pathways. We are grateful to our external advisory board of Patient Advocates and professional colleagues for their continuing advice and support.

**Figure 7.** An anticipated treatment plan for third line endocrine therapy. Patients must have responded and failed two successive antihormonal therapies to be eligible for a course of low dose estradiol therapy for 3 months. The anticipated response rate is 30%[89] and responding patients will be treated with anastrozole until relapse. Validation of the treatment plan via the Center of Excellence grant (Figure 5) will establish a platform to enhance response rates with apoptotic estrogen by integrating known inhibitors of tumor survival pathways into the 3 month low dose estrogen debulking treatment plan. The overall goal is to increase response rates and maintain patients for longer on antihormonal strategies before chemotherapy is required.

# 5

# Estrogen Receptors as Therapeutic Targets in Breast Cancer

*Eric A. Ariazi and V. Craig Jordan*

## 5.1
## Introduction

Breast cancer mortality has declined by 24% from 1990 to 2000, likely due to increases in the use of both mammography screening (followed by surgery) and adjuvant therapy, including chemotherapy and antihormonal tamoxifen (Figure 5.1) thera py [1]. Without screening and adjuvant therapy, it is estimated that deaths due to breast cancer would have risen by about 30% from 1975 to 2000. According to the consensus of seven models of reductions in the rate of death from breast cancer, decreases in mortality of 15% (median value) and 19% (median value) are due to mammography screening and adjuvant therapy, respectively. Endocrine therapy alone, most notably tamoxifen, a selective estrogen receptor (ER) modulator (SERM) that blocks estrogen action in breast cancer, is estimated to account for a 9.8% (median value) decrease in breast cancer mortality [1]. Additionally, the recent studies that connect hormone replacement therapy (HRT) with an elevation in breast cancer incidence has resulted in a fall by 8.6% in the annual age adjusted breast cancer incidence from 2001 to 2004 as women chose to stop long term HRT [2 4]. Still, the American Cancer Society estimates that in 2007, 178 480 American women will have been diagnosed with new cases of breast cancer and an estimated 40 460 women will have died from the disease, with only lung cancer being responsible for more women's cancer deaths [5]. Worldwide, it is anticipated that in the coming decade, 5 million women will be affected by breast cancer [6]. Clearly, further advances in the development of treatments, particularly ones with fewer undesirable side effects, are necessary.

Seminal work conducted by Elwood Jensen and reported in 1962 demonstrated that estrogen target tissues, such as the uterus, vagina and pituitary gland retain tritiated $17\beta$ estradiol (E2) (Figure 5.2) administered subcutaneously to immature rats, while nontarget tissues, such as the kidney, liver and muscle, do not ([7, 8], reviewed in Ref. [9]). This selective retention proved the existence of an ER in the target tissues. The receptor was isolated as an extractable protein from the rat uterus
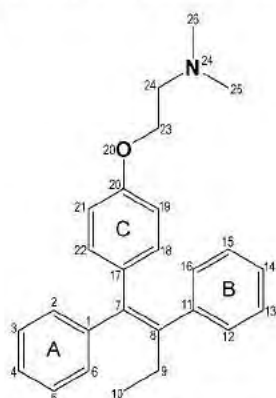
**Figure 5.1** Tamoxifen. Atoms are numbered and rings are designated by letters.

by Toft and Gorski in 1966 [10]. Jensen [11] reasoned that if ER were present in a breast tumor, then this would increase the probability of a response to endocrine ablative therapy (oophorectomy, adrenalectomy, hypophysectomy). Indeed, approxi mately 75% of breast cancers are positive for ER expression, for which the routine testing is used to predict response to antihormonal therapy [12].
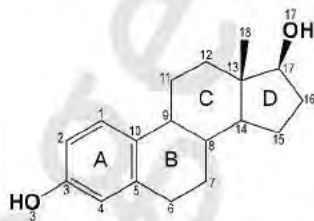


**Figure 5.2** E2. Atoms are numbered and rings are designated by letters.

ER was cloned and sequenced from MCF 7 human breast cancer cells 20 years after its purification in 1986 [13, 14]. This ER has since been renamed ERα (ESR1, NR3A1) (Figure 5.3), due to the cloning of a second ER in 1996, ERβ (ESR2, NR3A2) (Figure 5.3), from a rat prostate cDNA library based on its sequence similarity to ERα [15]. Human ERβ was subsequently cloned from a testis cDNA library [16]. ERs, member of the steroid/thyroid hormone nuclear receptor superfamily, bind estro gens with high affinity and regulate transcription in an estrogen dependent manner (reviewed in Refs [17 24]). While the classical genomic function of the receptor has been understood for some time, emerging evidence suggests that the receptor participates in a broader range of biological activities, including cross talk with other signal transduction pathways. ERα is the predominant ER expressed in breast cancer [25, 26] and the clinical significance of ERβ in breast cancer remains unclear (reviewed in Refs [23, 27, 28]). Hence, if not specified, ER refers to ERα.
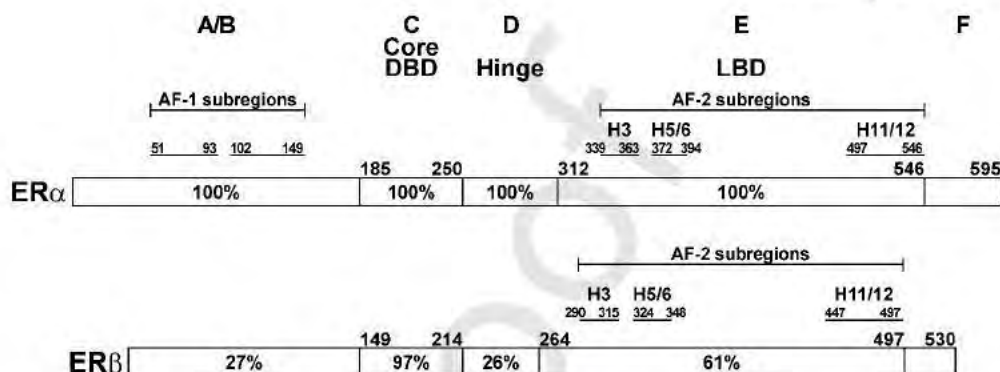
Figure 5.3 Domain structure and sequence identity between ERα and ERβ. Regions were subdivided in the A/B region (N terminus), core DBD (C region), D region (hinge), LBD (E region) and F region (extra C terminus). Comparisons were made using the Gap program (Genetics Computer Group). Numbers correspond to amino acids at the ends of the indicated region. Percentages in the bars correspond to the amino acid sequence identities. Open regions indicate no significant sequence identity. AF 1 subregions were defined according to mutant analysis [35]. The core DBD boundaries correspond to the 66 amino acids which define the zinc fingers responsible for sequence specific DNA interactions. The LBD boundaries correspond to the α helices H2 H12 and a single hairpin β sheet defined by the 1ERE crystal structure of human ERα complexed with E2 [42] and by the 1X7J crystal structure of human ERβ complexed with genistein [46]. The surface of AF 2 corresponds to the coactivator recognition groove formed by H3, H5/6, H11 and H12, which are defined according to ERα and ERβ crystal structures [42, 46].

The functional significance of ER in breast cancer has made it the foremost target, either directly or indirectly, for the development of antihormonal therapies aimed at the prevention and treatment of this disease. SERMs such as tamoxifen have been used to treat breast cancer successfully in the US since the late 1970s [29] and raloxifene (Figure 5.4), a related SERM, has completed testing as a chemopreventive against
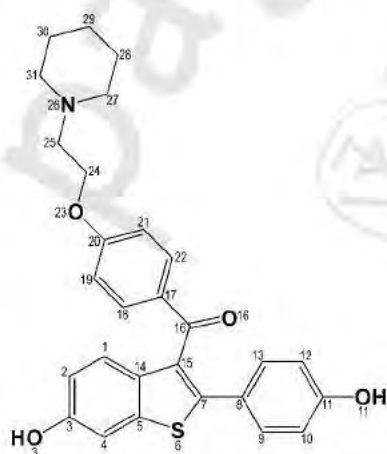


Figure 5.4 Raloxifene. Atoms are numbered.

tamoxifen in the Study of Tamoxifen and Raloxifene (STAR) [30]. We discuss these and the pure antiestrogen fulvestrant currently used for the treatment of breast cancer.

## 5.2
## Biology of ERs

### 5.2.1
### ERα and its Transcriptional Activation

The ERα gene is located on chromosome 6q25.1 and encodes a 595 amino acid, 66 kDa protein [31] composed of six functional regions (Figure 5.3) [32, 33]. The N terminal A/B region contains the ligand independent and functionally minor activating function (AF) 1 domain [34 36]. The C region, or the DNA binding domain (DBD), consists of a 66 amino acid motif that forms two structures termed zing fingers that interact with DNA, thereby mediating the receptor's sequence specific binding to estrogen response elements (EREs) found in the promoters of estrogen responsive genes [32, 33, 37, 38]. The D region, or hinge domain, contains the nuclear localization signal and interacts with heat shock factors. The E region, or ligand binding domain (LBD), interacts with E2 (Figure 5.2) as well as a diverse array of other compounds, and overlaps with the ligand dependent and functionally major tran scriptional AF 2 domain [23, 34, 36, 39 41]. The LBD consists of α helices H2 H12 and a single hairpin β sheet that, when complexed with E2, forms a canonical three layered antiparallel α helical sandwich structure similar to that observed in other nuclear receptors [42 46]. The C terminal region of the receptor is termed the F domain and inhibits dimerization of the receptor until it is bound by ligand [47]. In women, ERα is expressed in the mammary gland, uterus, vagina, ovary, bone, brain, cardiovascular system and liver [22].

ER's classic function in response to estrogen binding is genomic. ERα's AF 2 activities are largely regulated by the specific ligand occupying the LBD. In the nucleus, unliganded monomeric ERα exists as a complex with heat shock proteins (HSPs) (reviewed in Ref. [48, 49]). E2, a hydrophobic molecule, readily diffuses across the plasma and nuclear membranes. Once in the nucleus, E2 binds the LBD of the ERα HSP complex, leading to the disassociation of the HSPs. The LBD of the receptor then undergoes a crucial conformational change in which H12 covers the ligand binding pocket (LBP), and the receptor homodimerizes with another ERα molecule along surfaces in the LBD and DBD (reviewed in Refs [50, 51]). The DBDs of ERα proteins allow the homodimer to interact with EREs in the promoters of E2 responsive target genes [38]. The consensus palindromic ERE consists of two inverted half site repeats of AGGTCA separated by 3 nucleotides, to which ERα binds as a dimer with one unit each interacting with a half site. The AF 2 then mediates recruitment of the transcriptional machinery and in a cell type dependent fashion interacts synergistically with AF 1 region, to regulate transcription.

In ERα's LBD [42 44, 52], H2 H11 and the hairpin β sheet form a 'wedge shaped' hydrophobic ligand binding cavity, while H12, in the agonist bound conformation,
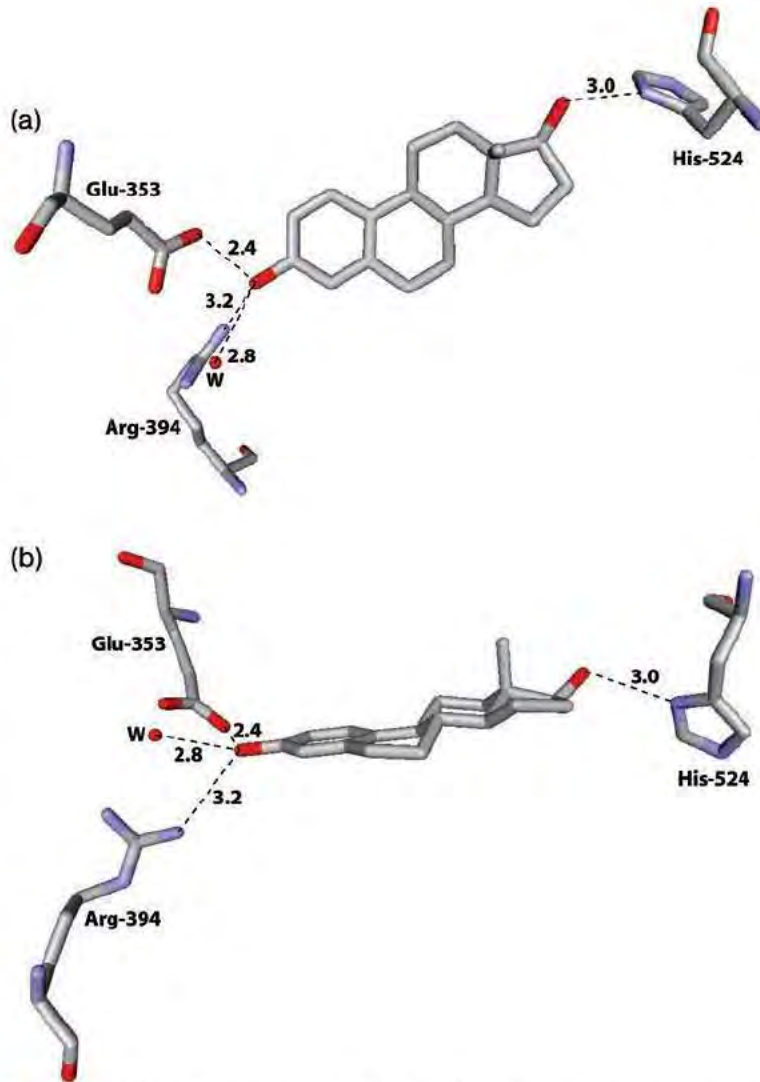
**Figure 5.5** Hydrogen bond interactions between E2 and ERα. Hydrogen bond intermolecular interactions of E2 cocomplexed with human ERα LBD using the X ray crystallographic structure 1GWR at 2.4 Å resolution [42] are shown from a top view (a) and a side view (b). The conformations are visualized using 3D Mol Viewer (a component of Vector NTI Advance 10.0.1 software; Invitrogen). Hydrogen bonds are indicated by dashed lines and their lengths in angstroms as determined by 3D Mol Viewer. A highly ordered water molecule stabilized by a hydrogen bond network is indicated (W). Carbon atoms are shown in gray, oxygen atoms in red and nitrogen atoms in blue.

closes over the cavity filled with E2 as if a 'lid'. E2 is aligned in the binding cavity by hydrogen bonds at both ends of the ligand (Figure 5.5); specifically the 3 OH group at the A ring end of E2 forms a strong hydrogen bond network with ERα's Glu353 (in H3) and Arg394 (in H6) as well as an ordered water molecule, while E2's 17β OH

group at the D ring end of the ligand hydrogen bonds with ERα's His524 (in H11). Further, hydrophobic van der Waals contacts along the lipophilic rings of E2, and particularly between Phe 404 and E2's aromatic A ring, promote a low energy conformation. Once E2 binds the receptor, H3, H5/6 and H11 form a groove to which H12 packs closely, orientating its hydrophobic surface towards the ligand and its charged surface away from the body of the LBD. Hence, H12 'seals' the ligand binding cavity and, together with H3, H5/6 and H11, forms as a highly complemen tary topology that defines the AF 2 surface (Figure 5.3) by interacting with coacti vators to promote transcriptional transactivation [42 44, 52].

Docking of coactivators is mediating by nuclear receptor boxes consisting of an LXXLL like motif present with the AF 2, in particular H12, and coactivators. Once docked to ERs (Figure 5.6), the core coactivator [e.g. steroid receptor coactivator 3 (SRC 3), also known as amplified in breast cancer 1], or the coactivator that directly interacts with ERs, recruits co coactivators into a multiprotein complex such as p300/CBP histone acetyltransferase, CARM1 methyltransferase, and ubiquitin ligases UbC and UbL. These coactivator complex proteins perform subreactions within the DNA ER coactivator complex necessary for transcription to proceed such as remodeling chromatin through methylation and acetylation modifications. They also direct their enzymatic activity towards adjacent factors, executing methylations and acetylations which promote dissociation of coactivator complex components, followed by ubiquitination to selectively target components of the complex to the proteasome for degradation after they have completed their functions, thereby allowing the next cycle of coactivator receptor DNA interactions to proceed (re viewed in Ref. [53]). Thus, an orderly yet dynamic sequence of complex assembly and disassembly ensues, culminating in transcription of estrogen responsive target genes (see Figure 5.6).

ERs also act through a tethered pathway of protein protein interactions at AP 1 sites [54 56], Sp1 sites [57 61] and NFκB sites [51]. At AP 1 sites, E2 activates ERα mediated transcription [54, 56]. Tamoxifen also activates AP 1 regulated transcrip tion via ERα in endometrial cells, but not in breast cells [56]. Thus, tamoxifen's effects on AP 1 activity are cell type dependent. Regarding ERβ, estrogens do not activate ERβ mediated transcription via AP 1 elements, but antiestrogens do [54]. Hence, when bound to estrogens, ERα and ERβ display opposing activities at AP 1 elements. However, there exist conflicting reports regarding whether E2 bound ERα up or downmodulates AP 1 regulated transcription [56, 62]. It is therefore likely that ERα differentially regulates transcription via AP 1 sites depending on the specific se quence of the AP 1 site, its context (i.e. surrounding sequences) and the cell type.

The ligand independent activation of ER via the AF 1 domain is closely related to the phosphorylation status of the receptor [63 65]. In particular, phosphorylation of Ser104/Ser106, Ser118 and Ser167 [23] has been identified. In U2OS human osteosarcoma cells; Ser104 and Ser106 are phosphorylated by the cyclin A cyclin dependent kinase 2 complex [66]. In multiple cell lines, Ser118 is phosphorylated in response to various treatments, including estrogens and antiestrogens, phorbol 12 myristate 13 acetate (PMA, also known as tetradecanoylphorbol acetate), epidermal growth factor (EGF) and insulin like growth factor 1 (IGF 1); the mitogen activated
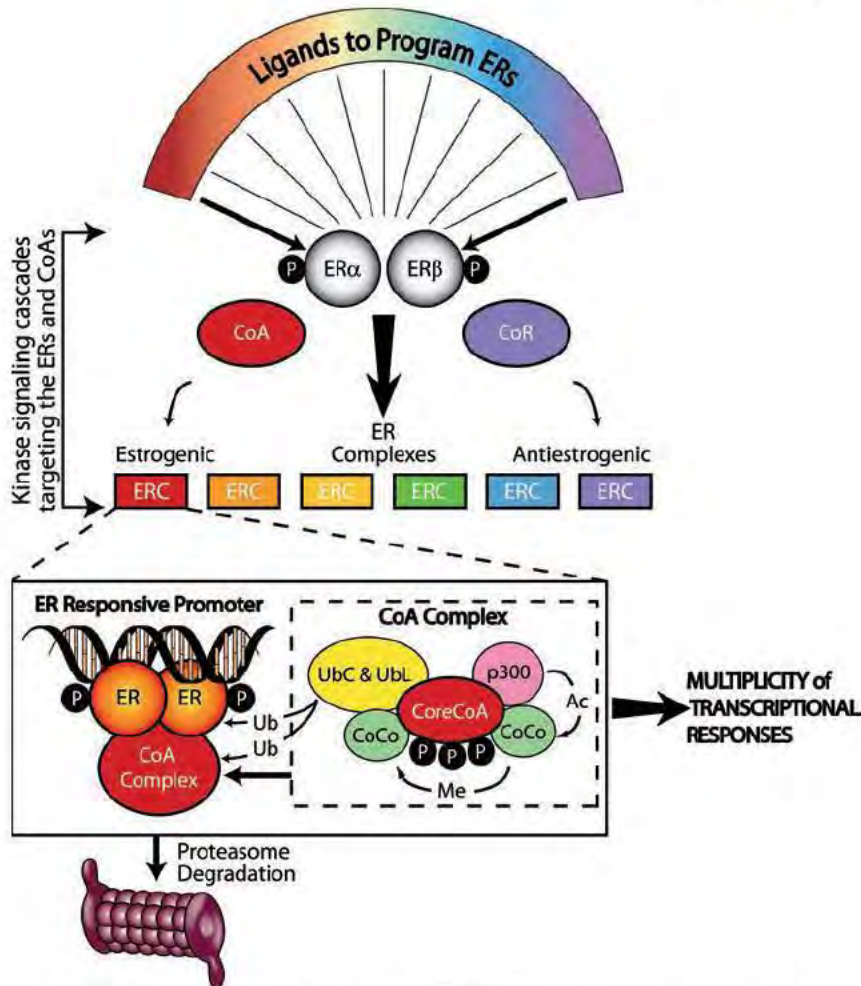
**Figure 5.6** Regulation of ER transcriptional activity in a target tissue. The shape of the ligand that binds to the ERs α and β programs the receptor to become an estrogenic or antiestrogenic signal by regulating the balance of coactivators (CoAs) and corepressors (CoRs) that are recruited to the receptors. Further, kinase signaling pathways target ERs and coactivators for phosphorylation, which then influences their ability to form complexes. Hence, a promoter bound by a ligand ER coregulator complex (ERC) containing significantly more coactivators than corepressors may be a dominant estrogenic site. However, the regulation of ER action is not simply due to the binding of the ligand ER coregulator complex to the promoter of the responsive gene, but a dynamic process of receptor complex assembly and destruction. A core coactivator facilitates assembly of an activated multiprotein complex containing specific co coactivators (CoCo) that may include p300/CBP histone acetyltransferase, CARM1 methyltransferase, and the ubiquitin conjugating ligases UbC and UbL. These co coactivators then acetylate (Ac), methylate (Me) and ubiquitinate (Ub) specific residues in the complex to remodel chromatin and to induce dissociation and destruction of receptor complex components via the proteasome. Thus, a regimented cycle of complex assembly, activation and destruction occurs based on the preprogrammed ER complex. Further, the target tissue is programmed to express a spectrum of responses between full estrogen action and antiestrogen action based on the shape of the ligand and the sophistication of the cell type specific coregulators.

protein kinase and Cdk7 have been implicated as the kinases responsible [65, 67 71]. Ser167 is phosphorylated in various cell lines by Akt, pp90$^{rsk1}$ and casein kinase II in response to treatment with E2, EGF or PMA [72 76]. Kinase signaling cascades also target coregulators for phosphorylation, which regulates their ability interact with ERs [77 79]. Overall, posttranslational modification of ER by phosphorylation modulates many facets of its activity including ligand, DNA and coregulator interactions [80].

### 5.2.2
### ERβ

The ERβ gene is located on chromosome 14q23.2 [81] and encodes a 530 amino acid protein (Figure 5.3) [82]. ERβ's DBD and LBD share the highest degree of amino acid identity, 97 and 61%, respectively, with the corresponding regions of ERα. However, the A/B and D domains only share 27/26 and 61% amino acid identity, respectively. Consistent with the lack of A/B domain homology between ERα and ERβ, functional studies have indicated that ERβ lacks AF 1 activity [83, 84]. ERβ is expressed in the testis, prostate, ovary, developing uterus, breast, vascular endothelium, smooth muscle, immune system, bone and some neurons (reviewed in Refs [22, 24]). The concentrations of ERα and ERβ vary according to the tissue, and even according to cell type within in a specific tissue. For example, ovaries express ERα and ERβ, but ovarian granulosa cells express exclusively ERβ [81].

Studies employing breast cancer cells demonstrated that ERβ antagonizes the proliferative effects of ERα [28, 85 88]. In MCF 7 cells, ERβ repressed expression of growth promoting genes c *myc*, cyclin D1, and cyclin A, while increasing expression of growth inhibitory genes p21$^{cip1}$ and p27$^{kip1}$, thereby leading to arrest in the G$_2$ phase of the cell cycle [85]. ERβ exhibits decreased transcriptional activity relative to ERα [83, 84, 89], due to impairment by ERβ's N terminal AF 1 [90]. This reduced transcriptional activity of ERβ may inhibit ERα transcriptional activity by competition for EREs and by formation of ERα ERβ heterodimers [84, 91, 92]. Five isoforms of ERβ exist, with the possibility of unique functions associated with specific isoforms. One of the more characterized isoforms, ERβ2, also termed ERβcx, results from alternative splicing in which 61 amino acids of the C terminal portion of the LBD and the entire AF 2 is replaced by a unique 26 amino acid sequence [93]. ERβ2 antag onizes ERα transcriptional activity via EREs and E2 responsive AP 1 sites, and promotes proteasome dependent degradation of ERα [94]. ERβ also opposes ERα transcriptional activity at specific promoters through AP 1 sites by altering the recruitment of c Fos and c Jun to E2 responsive AP 1 sites in promoters [95]. For example, ERβ blocks ERα transcriptional activity at the cyclin D1 promoter [96]. However, ERβ does not always inhibit ERα transcriptional activity; genome wide analysis of MCF 7 cells overexpressing ERβ demonstrated that ERβ enhances or represses distinct subsets of estrogen regulated genes [97]. Interestingly, ERβ regulated expression of genes which may contribute to suppression of growth 9i.e. components in the transforming growth factor β pathway), and genes control ling cell cycle progression and apoptosis [97].

The function of ERβ and its role in breast cancer remains controversial. ERβ is coexpressed with ERα in around 60% of unselected primary breast cancers and in around 50 60% of ERα negative breast cancers [98 100]. Recent studies have shown that ERβ expression is highest in normal breast and progressively declines through ductal usual type hyperplasia to ductal carcinoma *in situ* to invasive breast carcinoma [87, 98, 101]. In ductal usual type hyperplasia, low levels or ERβ compared to ERα predicted progression to invasive breast carcinoma [102]. In malignant disease, high ERβ protein levels predicted improved disease free and overall survival in patients treated with adjuvant tamoxifen [103], particularly in tamoxifen treated patients with ERα negative disease [104]. Other studies found that coexpression of ERβ and ERα mRNA associated with node positive disease [105], that increased ERβ mRNA levels associated with tamoxifen resistance [106] or that ERβ mRNA expression did not add significant value to predicting response to neoadjuvant SERM therapy [107]. Con sidering the potential for prognostic value of specific ERβ isoforms, ERβ2 protein levels associated with favorable response to endocrine therapy [108]. The splicing variant ERβ2 has also been observed to correlate with progesterone receptor (PR) negativity in ERα positive breast cancer, possibly as a result of repressing ERα mediated induction of PR expression [109]. If expression of ERβ, or specific isoforms of ERβ, proves to be an important prognostic factor for breast cancer, breast tumors will need to be tested routinely for ERβ in the same way that they are now tested for ERα [110, 111].

In summary, the role of ERβ in breast pathobiology remains unclear. However, the majority of recent studies suggests that ERβ expression has a potential growth inhibitory effect on normal and neoplastic breast cells and could represent a favorable prognostic factor in breast cancer (reviewed in Ref. [23, 27, 28]). A better under standing of the role of ERβ in breast and possibly other forms of cancer could elucidate ways of exploiting it as a therapeutic target using ERβ selective ligands. ERα and ERβ selective ligands are addressed elsewhere in this book (Chapter ■XX■).

## 5.3
### Therapeutic Basis for Targeting ER

Blocking estrogen synthesis using aromatase inhibitors (AIs) is therapeutically successful for the adjuvant treatment of breast cancer and is considered to be superior to adjuvant tamoxifen treatment with fewer side effects, such as endome trial cancers, hysterectomies and blood clots [112 115]. There are two classes of agents to prevent the CYP19 aromatase enzyme from synthesizing estrogen: a competitive inhibitor can be employed (e.g. letrozole or anastrozole) or a suicide inhibitor can be used (e.g. exemestane) [116]. This indirect method of targeting the tumor ER is unfortunately too large a topic to cover adequately in this chapter so the interested reader is referred to the clinical and translational articles mentioned above for further information. We have chosen instead to focus our chapter on compounds that target ER directly. The nonsteroidal compounds that bind to ER and

modulate the signal transduction pathway at different target sites around the body are called SERMs. In contrast, a group of steroidal compounds bind to ER and cause rapid destruction of the complex. These compounds are called 'pure antiestrogens' as they exhibit no estrogen like actions at sites around the body.

The administration of estrogenic compounds as HRT ameliorates many of the symptoms of menopause, including hot flashes and night sweats, in addition to reducing the risk of colon cancer and osteoporosis/fractures (reviewed in Ref. [117]). Unfortunately, HRT also increases the risk of Alzheimer's disease, strokes, blood clots, breast cancer and reduced cognitive function. This combination of effects in multiple tissues illustrates the importance of selectivity in the modulation of ER for the treatment of breast cancer. SERMs, such as tamoxifen, its active metabolites and raloxifene, function as partial antagonists depending on tissue and promoter context. For example, both tamoxifen and raloxifene function as antagonists in mammary tissues, and as agonists in bone, brain and cardiovascular tissues (reviewed in Ref. [118]). The effects of the two SERMs differ in uterine tissue where raloxifene exhibits antagonistic activity, but tamoxifen exhibits partial agonistic activity thought to be associated with an increased risk of endometrial cancer [119 123]. An ideal SERM would decrease the incidence of osteoporosis, coronary heart disease, hot flashes and breast cancer without increasing the risk of blood clots and endometrial cancer (reviewed in Ref. [117]). We will provide a basic background of the current direct utility of the two pioneering SERMs tamoxifen and raloxifene, discuss in detail the putative mechanism of action of SERMs, consider progress with new SERMs, and close with an examination of pure antiestrogens.

## 5.4
## SERMs

### 5.4.1
### Origins of SERMs

It is important to remember that tamoxifen was discovered in a fertility control program at a time in the late 1950s/early 1960s when interest by the pharmaceutical industry was focused on contraception [124, 125]. Similarly, raloxifene, then known as keoxifene, was designed to be a potential therapy for the treatment of breast cancer, but it failed in that application during the 1980s [126]. By coincidence, both 'nonsteroidal antiestrogens' [127] were subsequently reinvented for use as their current clinical applications: tamoxifen as a targeted, long term breast cancer therapy and chemopreventive, and raloxifene as a target tissue specific modulator of estro genic and antiestrogenic actions. Raloxifene was found to prevent bone loss in ovariectomized rats [128], while the same doses prevented rat mammary carcino genesis [129]. It was clear that the drug group, now referred to as SERMs could switch on and switch off estrogen target sites around the body. It was also apparent that based on the fact that tamoxifen and raloxifene could potentially maintain bone density, but prevent breast cancer, that the drugs could be used to prevent osteoporosis in

postmenopausal women and prevent breast cancer as a beneficial side effect [130]. Since raloxifene did not appear to have the same stimulating effects in the rodent uterus and human endometrial cancer as tamoxifen [131, 132], it was the obvious candidate for development by the pharmaceutical industry. Others would follow and these compounds will be discussed later in this chapter. It also became pertinent to consider the mechanism of action of SERMs because the application of these compounds grew broader than just targeting ER in breast cancer. The recognition of target site specific effects of SERMs naturally initiated an investigation of their molecular mechanisms of action because the traditional model of estrogen action with estrogens binding to ER to initiate responses and 'antiestrogens' blocking estrogen induced events was no longer consistent with the facts. The SERMs became new pharmacology tools to explore the target site specific actions of ER.

### 5.4.2
### Currently Approved SERMs Tamoxifen and Raloxifene

#### 5.4.2.1 Tamoxifen

Tamoxifen (ICI 46,474; Nolvadex®; Figure 5.1) was reported in 1967 as a possible contraceptive, but Arthur Walpole of the ICI Pharmaceuticals Division had the foresight to include in its patent application a use for the 'control of hormone dependent tumors' despite the fact that no studies had, at that time, been completed [133, 134]. During the 1970s, tamoxifen was reinvented as a drug targeted to ER, and used strategically as a long term adjuvant therapy for the treatment and prevention of breast cancer. In the US, tamoxifen received Food and Drug Administration (FDA) approval as an adjuvant treatment for node positive breast cancer in postmenopausal women with chemotherapy in 1985 and alone in 1986. FDA approval for its use for the treatment of ER positive advanced breast cancer in premenopausal women came in 1989, and approval as an adjuvant treatment for node positive ER positive breast cancer in pre and postmenopausal women came in 1990 [118]. Tamoxifen is also the first drug FDA approved for chemoprevention of breast cancer incidence in high risk pre and postmenopausal women [135]. Tamoxifen was considered the standard of care for the treatment of ER positive breast cancer as recommended by the 2000 US National Institutes of Health Consensus Development Conference and the 2001 St Gallen Consensus Panel [136], and is credited with saving the lives of 400 000 breast cancer patients while maintaining bone mineral density.

A 5 year course of tamoxifen treatment provides protection superior to 1 2 years of treatment. Currently, 5 years of adjuvant tamoxifen is recommended to be optimal, since extending treatment beyond 5 years provides no further improvement [137, 138]. There are reports of tamoxifen stimulated tumor growth occurring during the treatment of advanced (metastatic stage IV) breast cancer [139, 140], but there is currently no evidence that extending tamoxifen beyond 5 years of adjuvant therapy increases the risk of tumor recurrence. Critically important, the protective effects of tamoxifen on breast cancer recurrence and mortality are persistent long after tamoxifen therapy is stopped. A meta analysis of 15 years of follow up of 10 386 women shows that 5 years of adjuvant tamoxifen in ER positive disease versus not

almost halves the annual recurrence rate (recurrence rate ratio $= 0.59$, SE $= 0.03$) and decreases mortality by a third (death rate ratio $= 0.66$, SE $= 0.04$). These decreased rate ratios translate into 15 year gains of 11.8 (1.3) and 9.2% (SE $= 1.2$) in recurrence and mortality, respectively [141]. However, tamoxifen is not an ideal SERM because it increases the incidence of hot flashes, vaginal discharge, blood clots and endometrial cancer (reviewed in Refs [118, 136, 142, 143]). After 10 years of clinical reporting (1989 1999), it appears that tamoxifen causes a 3 to 4 fold increase in endometrial cancer in postmenopausal patients, although it should be noted that the absolute risk of developing such cancer is low. However, there is no association between tamoxifen use and endometrial cancer risk in premenopausal women [135, 144, 145], probably because menstrual cycles persist during tamoxifen treatment for the majority of women. Thus, current practice outside the clinical trial setting with tamoxifen is a 5 year course of treatment with regular monitoring for endometrial cancer [136].

In the 1960s, it was observed that the *E* and *Z* isomers of substituted tripheny lethylenes exerted opposing biological activities [146]. Tamoxifen (ICI 46,474) is the *Z* isomer of *p* dimethyaminoethoxy 1,2 diphenylbut 1 ene (Figure 5.1) and is an anti estrogen in the rat [134, 146]. In contrast, ICI 47,699 (Figure 5.7) was confirmed as the *E* isomer by X ray crystallography and is an estrogen [147, 148].
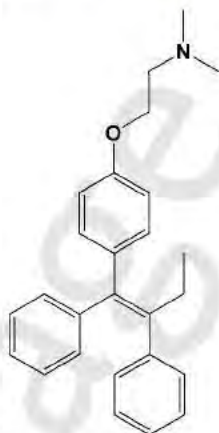


**Figure 5.7** ICI 47,699.

Tamoxifen is metabolized to its major metabolite the weak antiestrogen *N* desmethyltamoxifen (Figure 5.8) by cytochrome P450 3A4/5 (CYP3A4/5) enzymes, and to the minor yet potent antiestrogen 4 OH tamoxifen (Figure 5.9) by CYP2D6 and other P450s, as well as to other minor metabolites [127, 149 154]. The abundant metabolite *N* desmethyltamoxifen undergoes secondary metabolism by CYP2D6 into the major secondary metabolite 4 OH *N* desmethyltamoxifen, or endoxifen (Figure 5.10) [149, 155], and by the CYP3A subfamily to additional secondary metabolites [149, 155]. Also, 4 OH tamoxifen undergoes secondary metabolism by the CYP3A subfamily to endoxifen [149]. For decades, 4 OH tamoxifen was presumed to be the most important active metabolite of tamoxifen,

**Figure 5.8** *N* Desmethyltamoxifen.



**Figure 5.9** 4 OH tamoxifen.



**Figure 5.10** Endoxifen.
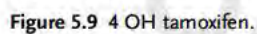
because tamoxifen has a low binding affinity for ER, but 4 OH tamoxifen exhibits a high binding affinity for ER equivalent to that of E2. Further, 4 OH tamoxifen is a potent antiestrogen in the rat [151, 156]. Thus, tamoxifen is a prodrug that is converted to the active metabolite 4 OH tamoxifen *in vivo* [151, 157]. However, recent evidence shows that endoxifen is also a potent metabolite: both 4 OH tamoxifen and endoxifen bind ERα and ERβ with similar affinity, and inhibit E2 induced proliferation of human breast cancer cells with similar potency [158]. Also, 4 OH tamoxifen and endoxifen regulate global gene expression similarly [159]. Since the average plasma concentration of endoxifen is 5 to 10 fold higher than that of 4 OH tamoxifen in breast cancer patients administered tamoxifen chronically [160], endoxifen is the major active metabolite, yet the combined concentrations of both endoxifen and 4 OH tamoxifen likely determine the total antiestrogenic activity of tamoxifen *in vivo*.

Plasma concentrations of endoxifen are significantly influenced by CYP2D6 genotype, which exists as wild type (functional alleles) or variant alleles that exhibit reduced functionality or are nonfunctional [161]. The CYP2D6 genotype homozygous for a nonfunctional allele (*4/*4) results in low endoxifen levels [162], and predicts a higher risk of disease relapse and lower incidence of hot flashes [163, 164]. These studies suggest that hot flashes, a side effect thought to be due to the antiestrogenic actions of tamoxifen, may be an indirect measure of CYP2D6 activity. In support of this hypothesis, a recent study showed that hot flashes compared to no hot flashes served as an independent predictor of tamoxifen efficacy by associating with relapse free survival [165]. CYP2D6 can be potently inhibited by paroroxetine, a selective serotonin reuptake inhibitor, which is often prescribed to individuals taking tamoxifen to alleviate hot flashes. Indeed, patient's coadministered paroxetine with tamoxifen exhibit lower levels of endoxifen than patients not coadministered paroxetine [155, 166]. Further, in a prospective clinical trial involving breast cancer patients chronically treated with tamoxifen, individuals exhibiting: (i) low endoxifen/*N* desmethyltamoxifen ratios associated with the CYP2D6 genotype lacking any functional allele, (ii) intermediate endoxifen/*N* desmethyltamoxifen ratios with at least one functional allele and (iii) high endoxifen/*N* desmethyltamoxifen ratios with two or more functional alleles [162]. Therefore, endoxifen plasma levels, affected by the CYP2D6 genotype and CYP2D6 inhibitors, may impact response to tamoxifen therapy [161].

Tamoxifen treatment increases a number of estrogen induced circulating proteins, such as sex hormone binding globulin [167, 168] and antithrombin III [168, 169], and alters the plasma protein profile [170, 171]. Additionally tamoxifen has an estrogen like action to reduce luteinizing hormone (LH) and follicle stimulating hormone (FSH) in postmenopausal women [172]. Tamoxifen has a consistent ability to decrease low density lipoprotein (LDL) cholesterol, but unlike estrogen does not cause an increase in high density lipoprotein (HDL) cholesterol [169, 173 177]. Although tamoxifen was originally classified as an antiestrogen, the drug does not predispose women to coronary heart disease [178 180]. Most studies find that tamoxifen does not protect against coronary heart disease, but the finding may be because only clinical trials with small numbers of patients at risk have been examined. Only retrospective results from the Scottish adjuvant tamoxifen trial of

5 years of adjuvant tamoxifen showed a decrease in fatal myocardial infarc
tion [181, 182]. Tamoxifen has not been tested prospectively for the prevention of
coronary heart disease in high risk women. Tamoxifen maintains bone density in
postmenopausal women [183  186] and causes a slight decrease in bone density in
premenopausal women [187]. The drug has not been tested prospectively as a
preventive for osteoporosis, but a nonsignificant decrease in hip, wrist and spinal
fractures has been noted as a secondary endpoint in the National Surgical Adjuvant
Breast and Bowel Project chemoprevention trial [135]. Interestingly enough, tamoxi
fen produces significantly fewer fractures compared to AIs when used as an adjuvant
therapy in postmenopausal women [188  190]. Although tamoxifen could be classi
fied as a partial agonist in most estrogen like parameters, the reduced estrogenicity is
not reflected in a reduction in the incidence of blood clots relative to hormone
replacement therapy HRT [135, 191].

Tamoxifen had been used for more than a decade for the treatment of breast cancer
(in Europe since the early 1970s and in the US since 1978) without the reporting of
serious side effects [127]. However, by the end of the 1980s, with the expanded use of
tamoxifen as a long term adjuvant therapy in node negative breast cancer [192] and
the proposed use of tamoxifen for chemoprevention in high risk women [193], there
was a requirement to reexamine the toxicology of tamoxifen in greater detail.
Tamoxifen was found to initiate hepatocellular carcinoma in rats by a non ER
mediated mechanism [194  198]. This finding was a major concern and naturally was
linked to an increased incidence of endometrial cancer and two cases of hepatocel
lular carcinoma noted in women taking tamoxifen [122, 199, 200]. The laboratory
finding of carcinogenicity, so late in the drugs' development, occurred because the rat
had not previously been used to evaluate the long term toxicology of tamoxifen prior
to introduction as a breast cancer treatment. This was not a requirement. It was
equally true that if tamoxifen had been tested and found to be carcinogenic, then
adjuvant endocrine therapy, AIs, SERMs and raloxifene would not have been pursued
without proof of principle that tamoxifen was a SERM and saved lives [199]. Needless
to say, tamoxifen induced liver carcinogenicity was thoroughly investigated. In rats
administered tamoxifen, DNA adducts accumulated in the liver and were identified
as $\alpha$ OH tamoxifen (Figure 5.11) covalently linked to the oxocyclic amino of
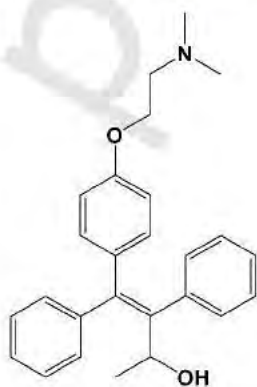


**Figure 5.11** $\alpha$ OH tamoxifen.

deoxyguanosine [201 205]. Although DNA adducts were readily identified in rat and mouse hepatocites (90 and 15 adducts per 108 nucleotides, respectively), DNA adducts were not detected in human hepatocytes following tamoxifen treatment [204]. Similarly, the pattern of DNA adducts found in the rat liver was not found in the liver obtained from patients treated with tamoxifen [206]. Overall, it appears that specific metabolic pathways in rat liver predispose that species to liver carcinogenesis [207]. α OH tamoxifen is a poorer substrate for human sulfotransferase (which is appar ently necessary for adduct formation [208]) than the rat form of the enzyme. Conversely, glucuronidation, which would detoxify α OH tamoxifen, predominates in human hepatocytes [209]. This area of drug evaluation is extremely important for understanding the relevance of species related toxicity to clinical practice. Phillips has reviewed the genotoxicity of tamoxifen [210]. In his conclusion he raises the concept of whether tamoxifen is a genotoxic carcinogen in the rat, but a nongenotoxic carcinogen in humans. This may make tamoxifen unique.

### 5.4.2.2 Raloxifene

The SERM raloxifene (Evista®, previously keoxifene and LY156758; Figure 5.4) is a failed breast cancer therapeutic since it showed either no activity or modest activity as a breast cancer therapy [211, 212]. Hence, drug development of raloxifene as an antitumor agent was abandoned in the late 1980s. These data are consistent with the laboratory finding that raloxifene is less effective than tamoxifen in animal models of breast cancer [129, 131]. The fact that raloxifene has extremely poor (2%) bioavail ability because of rapid first pass phase II metabolism [213] suggests that long acting agents are required for the treatment of breast cancer. However, the recognition of selective ER modulation and the possibility of developing multifunctional medi cines [117, 128, 133] has resulted in the successful development of raloxifene to treat and prevent osteoporosis [214]. The successful development of raloxifene is a direct result of a novel finding that nonsteroidal antiestrogens can maintain bone densi ty [128, 215, 216], but may not increase the risk of breast cancer like HRT [3, 4, 217]. Hence, raloxifene was approved in 1997 by the FDA for the prevention of osteoporo sis in postmenopausal women.

As a SERM, raloxifene exerts partial estrogen like action at specific target tissues. Estrogens are known to prevent bone mineral density loss; likewise, raloxifene has been conclusively shown to prevent bone loss and reduce the risk of vertebral fractures [218]. Preliminary studies in 251 normal postmenopausal women random ized into groups taking placebo, raloxifene (200 mg daily), raloxifene (600 mg daily) or Premarin™ (0.625 mg daily) show decreases in serum alkaline phosphatase, serum osteocalcin, urinary pyridinoline and urinary calcium excretion with raloxifene that were no different than with estrogen [219]. However, the doses of raloxifene were far higher than the 60 mg daily currently recommended for the prevention and treat ment of osteoporosis. Evaluation of raloxifene (60 mg daily) on bone remodeling in early postmenopausal women, using calcium tracer kinetic methods, found that although remodeling suppression was greater for estrogen, the remodeling balance was the same for the two agents [220]. These results are consistent with the finding that raloxifene increases bone density by $2.4 \pm 0.4\%$ in the lumber spine and

2.4 ± 0.4% for the total hip [221]. Raloxifene has also been shown to decrease spine fractures by 40%, although there was no significant decrease in hip fractures [222].

Raloxifene received a rigorous evaluation in the human uterus. In women pre screened to ensure the absence of preexisting endometrial abnormalities, raloxifene did not show an increase in endometrial thickness [221, 223 225]. Data from postmenopausal women showed that raloxifene was not associated with vaginal bleeding or an increased endometrial thickness [221, 226]. To date, raloxifene has not been associated with an elevated risk of endometrial cancer, but laboratory studies demonstrated that the drug will support the growth of a tamoxifen stimulated endometrial cancer transplanted into athymic mice [227, 228]. However, the growth response of human endometrial carcinoma to raloxifene under laboratory conditions was not as much as that of tamoxifen or toremifene [120].

On the basis of the hypothesis that raloxifene could reduce the incidence of breast cancer as a beneficial side effect of the prevention of osteoporosis [215], the placebo controlled trials with raloxifene have been monitored for changes in breast cancer incidence. One of the first studies to show this was the Multiple Outcomes of Raloxifene Evaluation (MORE) trial [226]. In this study, 7704 postmenopausal women (mean age of 66.5 years) with osteoporosis were randomized to receive one or two daily oral doses of raloxifene (60 mg) or placebo. Raloxifene decreased the relative risk (RR) of invasive breast cancer by 76% [RR = 0.24; 95% confidence interval (CI) = 0.13 0.44] during 3 years of treatment with raloxifene. raloxifene treatment resulted in a increase in the risk for venous thromboembolism (RR = 3.1; 95% CI = 1.5 6.2), but no increase in the risk of endometrial cancer was observed (RR = 0.8; 95% CI = 0.2 2.7) [226]. Subsequently, women who had been enrolled in the MORE trial were enrolled in the Continuing Outcomes Relevant to Evista (CORE) trial, where 3510 women who had received either dose of raloxifene in the MORE trial were assigned to receive 60 mg of raloxifene and 1703 women who had been assigned to receive placebo in the MORE trial continued on placebo. In this second trial, when compared to placebo, raloxifene reduced the 4 year incidences of invasive breast cancer and ER positive invasive breast cancer by 59% [hazard ratio (HR) = 0.41; 95% CI = 0.24 0.7] and 66% (HR = 0.34; 95% CI = 0.18 0.66), respectively. These differ ences were not observed in ER negative invasive breast cancer. When follow up for both the MORE and CORE trials were combined totaling 8 years, decreases of 66% (HR = 0.34; 95% CI = 0.22 0.50) and 76% (HR = 0.24; 95% CI = 0.15 0.40) in the incidence of invasive and ER positive breast cancers, respectively, were observed when comparing the raloxifene to the placebo arm. The increase of thromboembo lism (RR = 2.17; 95% CI = 0.83 5.70) was confirmed, but no new safety concerns related to raloxifene therapy were identified [229]. Hence, like tamoxifen, raloxifene appears to prevent breast cancer in high risk women, but unlike tamoxifen, has not been found to increase the incidence of endometrial cancer.

Raloxifene's effects on risk factors for coronary artery disease are similar to those of estrogen, by lowering LDL cholesterol and homocysteine levels. Blood clots with raloxifene also occur at the same frequency as observed with HRT. However unlike estrogens, it does not increase triglyceride, HDL cholesterol or C reactive protein levels [230]. Therefore, in addition to being evaluated for the prevention of

osteoporosis and breast cancer, raloxifene was evaluated for the reduction of the risk of coronary artery disease in the Raloxifene Use for The Heart (RUTH) trial. This study evaluated whether 60 mg/day of oral raloxifene reduced the risk of coronary events and risk of invasive breast cancer in 10 101 postmenopausal women with documented coronary heart disease or who are at high risk for developing it. Unfortunately, raloxifene had no effect on cardiovascular risk in the RUTH trial, but did serve to further confirm that raloxifene prevents invasive breast cancer and vertebral fracture with no increased risk of endometrial cancer in postmenopausal women [231].

These studies led to the trial that compared raloxifene to tamoxifen in the prevention of breast cancer in women who are at high risk of developing the disease: the STAR trial. This trial enrolled 19 747 postmenopausal women (mean age 58.5 years) at high risk of breast cancer to receive 5 years of either tamoxifen (20 mg daily) or raloxifene (60 mg daily). Raloxifene was demonstrated to be equally as effective as tamoxifen in reducing the incidence of invasive breast cancer (RR = 1.02; 95% CI = 0.82 1.28), while exhibiting a lower risk of thromboembolic events (RR = 0.70; 95% CI = 0.54 0.91) and cataracts than tamoxifen (RR = 0.79; 95% CI = 0.68 0.92) [30]. In contrast to tamoxifen, raloxifene was associated with a nonsignifi cant higher risk of noninvasive breast cancer (lobular and ductal carcinomas *in situ*). The mechanistic reason for why raloxifene may be less effective against noninvasive breast cancer is unknown. A lower incidence of uterine cancer was associated with raloxifene treatment (23 versus 36 cases in the raloxifene and tamoxifen groups, respectively), but this lower incidence was not statistically significant. However, the incidence of endometrial hyperplasia and hysterectomies was decreased in the raloxifene group compared to the tamoxifen group. Since raloxifene is already approved for prevention of osteoporosis, and it is equally as effective as tamoxifen for the prevention of invasive breast cancer with a lower incidence of side effects, raloxifene is poised to become a widely prescribed SERM.

## 5.5
### Mechanisms of Action of SERMS

### 5.5.1
#### Mechanism of SERM Antiestrogenic Action

Early studies described the interaction between the nonsteroidal antiestrogens and ER in the [³H]E2 ligand binding assay [232, 233], but the only conclusion that could be reached was that the compounds did bind to ER and block E2 binding. There was no ability to describe efficacy at ER without using assays *in vivo* using rats or mice [134, 146]. The ER assays did, however, help to identify tamoxifen as a prodrug that needed to be coactivated to a 4 OH metabolite to achieve potent antiestrogenic activity [151, 157]. Nevertheless, studies *in vivo* were not able to determine the actual biological activity at ER. At that time, the assay in the uterus or vagina was really the end result of drug metabolism and individual compounds were difficult to compare if

their pharmacokinetics were different. Only an assay *in vitro* could resolve many of the limitations in understanding the actual drug ER interactions.

Primary cultures of immature rat pituitary gland cells were first used to establish that nonsteroidal antiestrogens were competitive inhibitors of estrogen action and metabolic activation was an advantage but not a requirement for antiestrogen action [234]. The assay was used to describe the precise structure function relations for triphenylethylenes related to tamoxifen for the modulation of the prolactin synthesis through ER [127, 235] and to propose a precise region (the 'antiestrogenic region') necessary for the alkylaminoethyoxy side chain of antiestrogens to interact to prevent the correct folding of ER to develop full estrogen action. These data successfully translated to the study of growth regulation of ER positive breast cancer cells in culture once it was found that the cells were already grown in media containing fully estrogenic contaminants in the phenol red indicator [236]. Removal of the indicator and growth of cells in charcoal stripped serum (to remove estrogenic steroids) allowed an accurate description of the structure function relationships of estrogens and antiestrogens for the control of breast cancer cell growth [153, 237]. It was proposed that the side chain controlled the subsequent activation of ER inter acting with a hypothetical 'antiestrogen region' on ER. Changes in the side chain length [238] or basicity [239] were predicted to produce a range of complexes with different intrinsic activities that would result in different partial agonist activities [234, 235, 240].

Binding of SERMs such as 4 OH tamoxifen and raloxifene induces distinct conformations of ERα's LBD different from that of E2 [42, 44]. In a similar manner as E2, 4 OH tamoxifen and raloxifene bind within the same hydrophobic pocket. Also like E2, the phenolic hydroxyl group of 4 OH tamoxifen's A ring (Figure 5.12) and the hydroxyl group of raloxifene's benzothiophene moiety (Figure 5.13) are both posi tioned near H3 and H6, allowing formation of an hydrogen bond network with Glu353, Arg394 and an ordered water molecule. However, unlike E2, the antiestro genic side chains of 4 OH tamoxifen and raloxifene both protrude from the LBP between H3 and H11, making extensive hydrophobic contacts with these helices and interacting with Asp351. Due to the antiestrogenic side chains of 4 OH tamoxifen and raloxifene exiting the binding cavity adjacent to Asp351, H12 is displaced and does not cover the LBP. Rather, H12 assumes a conformation that mimics the position of the coactivator's nuclear receptor box motif, and occupies the coactivator recognition groove formed by H3, H4 and H5 [42, 44]. Therefore, the antiestrogenic properties of 4 OH tamoxifen and raloxifene are due in part to H12 not sealing the LBP and instead acting as an 'autoinhibitor' by preventing coactivator recruitment. This structural evidence confirmed earlier hypothetical models of estrogen and antiestrogen action [130], and provides an elegant solution to AF 2 silencing.

### 5.5.2
### Structural-Based Mechanisms of SERM Estrogen-Like Action

Although much progress has been made in our understanding of estrogen and antiestrogen action, there is no unifying theory that has explained the target
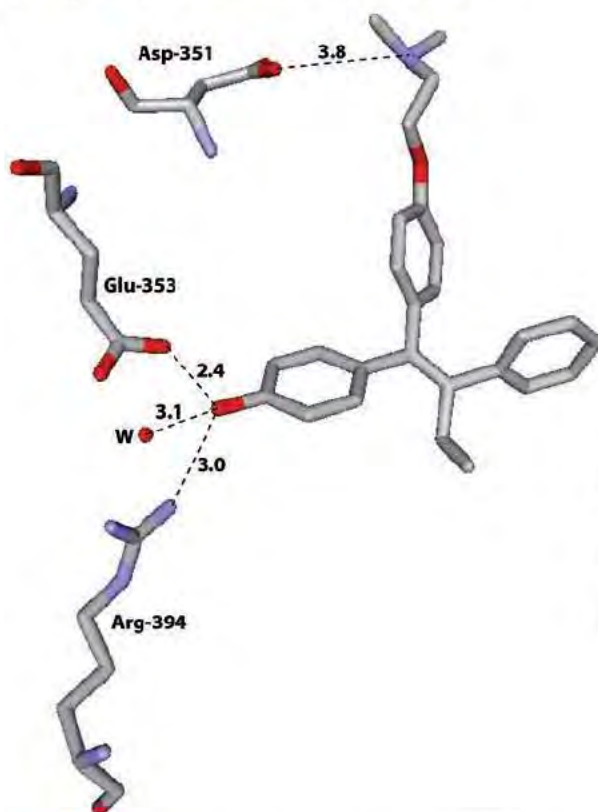
**Figure 5.12** Hydrogen bond interactions between 4 OH tamoxifen and ERα. Hydrogen bond intermolecular interactions of 4 OH tamoxifen cocomplexed with human ERα LBD using the X ray crystallographic structure 3ERT at 1.9 Å resolution [44] are shown. The conformation was visualized using 3D Mol Viewer as in Figure 5.5. Hydrogen bonds are indicated by dashed lines and their lengths in angstroms as determined by 3D Mol Viewer. A highly ordered water molecule stabilized by a hydrogen bond network is indicated (W). Carbon atoms are shown in gray, oxygen atoms in red and nitrogen atoms in blue.

site specific actions of SERMs. Despite this deficit, there are opportunities to imagine multiple mechanisms. In other words, there may be different mechanisms at different sites or groups of targets. By way of example, it is intriguing that raloxifene expresses less estrogen like activity than 4 OH tamoxifen in breast and uterine cells. The overall architecture induced by the SERMs in the LBD are similar enough that one could conclude that both raloxifene and 4 OH tamoxifen silenced AF 2. However, there are subtle differences between the positioning of the 4 OH tamoxifen and raloxifene in the LBD that ultimately affect the intrinsic activity of the SERM ERα complex. These clues now provide a link between the unusual pharmacology of the SERMs and the structure function relationships of their ERα complexes.

Chambon's group [40] was the first to address the issue of the target site estrogen like specificity of 4 OH tamoxifen using recombinant human ER. They reported
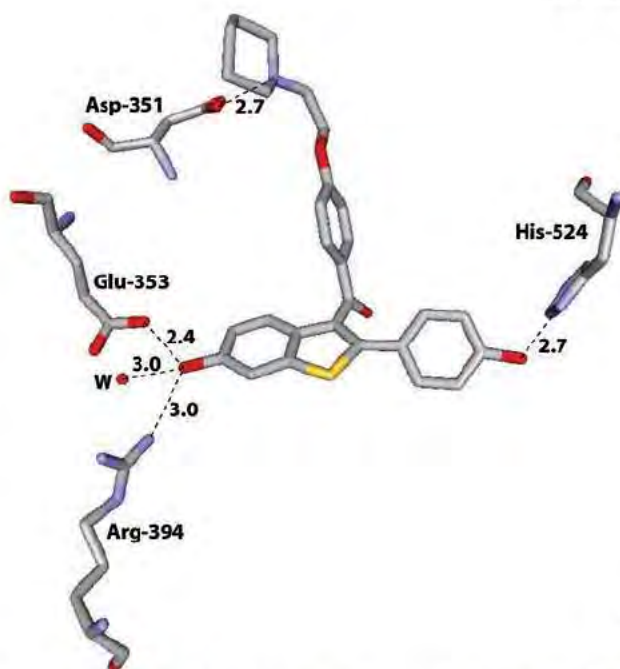
**Figure 5.13** Hydrogen bond interactions between raloxifene and ERα. Hydrogen bond intermolecular interactions of 4 OH tamoxifen cocomplexed with human ERα LBD using the X ray crystallographic structure 1ERR at 2.6 Å resolution [42] are shown. The conformation was visualized using 3D Mol Viewer as in Figure 5.5. Hydrogen bonds are indicated by dashed lines and their lengths in angstroms as determined by 3D Mol Viewer. A highly ordered water molecule stabilized by an hydrogen bond network is indicated (W). Carbon atoms are shown in gray, oxygen atoms in red, nitrogen atoms in blue and sulfur atoms in yellow.

that the estrogen like actions of 4 OH tamoxifen were cell type and promoter context dependent, which produced ligand independent activity of the AF 1 domain. This in turn, they stated, could explain the target site specific estrogen like actions observed with tamoxifen in animals and human tissue [127, 128]. In contrast, a pure antiestrogen (zero intrinsic activity) had no estrogen like actions in model systems or *in vivo* [40]. However, the fact that a pure antiestrogen could produce complete antiestrogenic activity by also silencing AF 1 suggested that AF 1 activity could be ligand specific, at least under controlled conditions.

A naturally occurring mutation of ER's amino acid 351 has provided valuable evidence that has led to demonstrating a precise interaction of amino acid 351 and the antiestrogenic side chain of a SERM, which in some way allosterically communicates with AF 1. The Asp351 → Tyr mutation was found in an unusual tamoxifen stimulated breast cancer tumor model [241]. Incidentally, this natural mutation is not responsible for tamoxifen induced drug resistance in patients. Asp351 is not involved in either the AF 1 or AF 2 regions, yet its mutation to Tyr converts the more antiestrogenic raloxifene to become an estrogen like compound. This was shown by measuring effects on expression of a relevant gene target *in situ*, transforming growth

factor α, in ER negative MDA MB 231 cells stably transfected with wild type and mutant ERα [242, 243]. It is important to note that this system also shows that 4 OH tamoxifen displays estrogenic like activity without the need for the Asp351 → Tyr mutation, underscoring that proper cellular context, such as in an ER negative breast cancer cell type, also plays a role in SERM mediated estrogenic activity. Subsequent X ray crystallography studies demonstrated that the antiestrogenic side chains of tamoxifen and raloxifene both exit the crystal structure of ERα adjacent to Asp351. However, the raloxifene piperidine ring nitrogen recognizes Asp351 through a hydrogen bond around 1 Å shorter than that of 4 OH tamoxifen's dimethylamino group (compare Figure 5.13 to Figure 5.12) [42, 44]. It has been hypothesized that raloxifene's piperidine ring both pushes H12 away (silencing AF 2 [42]) and shields or neutralizes the charge distribution around Asp351 more than that of 4 OH tamoxifen's diethylamino group. Since 4 OH tamoxifen interacts with Asp351 only weakly (3.8 Å distance; Figure 5.12), Asp351 instead takes on a position closer to H12. Conversely, raloxifene interacts more strongly with Asp351, thereby preventing Asp351 from positioning itself adjacent to H12. Thus, in the Asp → Tyr mutation, the distance between raloxifene's piperidine ring and amino acid 351 was increased, reflecting a weaker interaction, and instead, Tyr351 interacts with H12, which consequently prevents binding of corepressors and allowing estrogen like activity to manifest [244]. Additional amino acid substitutions lend further evidence in favor of this model. An Asp → Glu mutation also results in an increase in the distance between raloxifene's piperidine ring nitrogen and amino acid 351 from 2.7 Å in the wild type ER (Figure 5.13) to 3.5 5 Å in the mutant ER. This increased distance translated into an increase in estrogen like action of the raloxifene ERα com plex [245]. Removal of the charge at amino acid 351 with an Asp → Tyr substitution results in a loss of estrogen like properties [245]. The critical role of the intimate relationship between the antiestrogenic side chain of raloxifene and Asp351 is confirmed with the raloxifene derivative R1H where the piperidine ring of raloxifene is replaced by a cyclohexane. The ligand loses antiestrogenic properties and is a full agonist [121, 245]. Conversely, in the case of tamoxifen, an Asp → Gly351 mutation results in a decreased distance between tamoxifen's side chain and amino acid 351, leading to a tamoxifen ERα (Asp351 → Gly) complex that has lost estrogen like activity while retaining antiestrogenic properties [246, 247]. The Asp → Gly mutation also decreases the affinity of raloxifene for ERα, thereby illustrating the important role of the interaction of its piperidine side chain and Asp351.

McDonnell's group [248] has used a phage display technique to identify two separate coactivator binding sites responsible for the expression of the estrogen like effects of the E2 or tamoxifen ER complex. The coactivator binding site on the E2 ER complex could be the previously described AF 2 region, but the novel site on the tamoxifen ER complex could be the same as the transactivation site on ER referred to as AF 2b [246, 249]. The AF 2b site is more complex than the AF 2a site previously noted [250], which extends from amino acid 324 to amino acid 351. This is because acidic amino acids on H12 also play an essential regulatory role in the estrogen like action of tamoxifen. Mutations Asp538 → Ala/Glu542 → Ala/Asp545 → Ala in H12 reduce the intrinsic activity of the tamoxifen ER

complex [246, 249], thus indicating that the expression of SERM estrogen like actions requires interaction between amino acid 351 and H12, which together may define the occult SERM induced transactivation site AF 2b. Further, the full expression of SERM estrogen like actions requires synergistic allosteric interaction between AF 1 and AF 2b comprising amino acid 351 and H12. However, until the whole ligand receptor complex has been crystallized, it is not possible to visualize the relationship between AF 1 and AF 2.

### 5.5.3
### Coregulator-Based Mechanisms of SERM Estrogen-Like Action

Formation of an occult AF 2b transactivation domain induced by a SERM does not fully explain its tissue specific estrogenic activity. Other components in the ER signal transduction pathway, particularly coregulators that complex with the receptor, are crucial in determining cell type dependent properties of a SERM. It is reasonable to ask, how does the ligand program the receptor complex to interact with other proteins? X ray crystallography of the LBDs of ER liganded with either estrogens or antiestrogens demonstrates the potential of ligands to promote coactivator binding or prevent coactivator binding based on the shape of the estrogen or anti ER complex [42, 44]. Evidence has accumulated that the broad spectrum of ligands that bind to ER can create a broad range of ER complexes that are either fully estrogenic or antiestrogenic at a particular target site [251]. Thus, a mechanistic model of estrogen action and antiestrogen action has emerged based on the shape of the ligand that programs the complex to adopt a particular shape that ultimately interacts with coactivators or corepressors in target cells to determine the estrogenic or antiestrogenic response respectively (Figure 5.6).

It is more than a decade since the first steroid receptor coactivator (SRC 1) was described [252]. Not surprisingly, the coactivator model of steroid hormone action has now become enhanced into multiple layers of complexity, thereby amplifying the molecular mechanisms of modulation. It appears that coactivators are not simply protein partners that connect one site to another in a complex [253]. The coactivators actively participate in modifying the activity of the complex. Post translational modification of coactivators via multiple kinase pathways initiated by cell surface growth factor receptors (e.g. EGF receptor, IGF receptor 1 and ErbB2, also known as HER2) can result in a dynamic model of steroid hormone action. The core coactivator (e.g. SRC 3; Figure 5.3) first recruits a specific set of co coactivators, e.g. p300 and ubiquitin conjugating ligases, under the direction of numerous protein remodelers (e.g. the peptidyl prolyl isomerase Pin1, heat shock proteins and proteasome ATPases) to form a multiprotein coactivator complex that interacts with the phosphorylated ER at the specific gene promoter site [253]. Most importantly, the proteins assembled into the coactivator complex have individual enzymatic activities to acetylate or methylate adjacent proteins. This results in the dissociation of the complex and simultaneous tagging with activated ubiquitin. The activated ubiquitin is transferred to the ubiquitin conjugating enzyme that interacts with ubiquitin ligase which has already identified its protein target. Multiple cycles of

the reaction can polyubiquitinate a substrate (i.e. ER or a coactivator) or dependent on the ubiquitin ubiquitin linkage either can be activated further (K63 linkage), or degraded by the 26S proteasome (K48 linkage) [254].

Thus, for effective gene transcription, programmed and targeted by the shape and phosphorylation status of ER and coactivators, a dynamic and cyclic process of remodeling capacity is required for transcriptional assembly [255] that is immediately followed by the routine destruction of transcription complexes by the proteasome. Estrogen and SERM receptor complexes have differing accumulation patterns in the target cell nucleus [151, 256] primarily because the relative rates of destruction of the complexes are different [251].

These fundamental mechanisms [253, 257] in physiology can also be applied to the potential development of drug resistance to tamoxifen in breast cancer. Model systems have demonstrated the conversion of the tamoxifen ER complex from an antiestrogenic signal to an estrogenic signal in an environment enhanced for phosphorylation by overexpression of the ErbB2 cell surface receptor and an increase in SRC 3 coactivator accumulation [258, 259]. However, the enhanced level of coactivator and its enhanced phosphorylation state derived from an activated ErbB2 phosphorylation pattern will enhance the estrogen like activity of tamoxifen at ER. Clearly, issues of SERM action at target tissues and the eventual development of drug resistance in breast cancer will converge as duration of SERM use extends from a few years to at least a decade.

## 5.6
## Additional SERMs

### 5.6.1
### Clomiphene

In addition to tamoxifen [125, 134], clomiphene (originally chloramiphene or MRL 41; Figure 5.14) [260] resulted from a search for contraceptives in laboratory models,
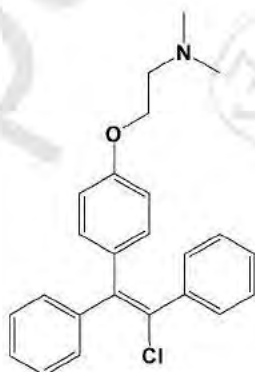


**Figure 5.14** Clomiphene.

but in clinical trials unexpectedly induced ovulation in subfertile women [261]. As a result of these and additional clinical findings, clomiphene [262 266] and initially tamoxifen [267, 268] were approved as profertility drugs for the induction of ovulation.

### 5.6.2
### Toremifene (Fareston®)

Toremifene (Figure 5.15), or chlorotamoxifen, has been thoroughly investigated in the laboratory [269 272] and has antitumor activity in carcinogen induced rat mammary cancer, but is less potent than tamoxifen [272 274]. Toremifene has been tested extensively in phase I III clinical trials [275 278] and has been approved for use in postmenopausal women with metastatic breast cancer [279]. As predicted from the reduced potency in animal studies, the dose required for activity is 60 mg of toremifene daily (tamoxifen is used at 20 mg daily). The side effects are similar to those of tamoxifen and, as with tamoxifen, the responses are observed in ER positive tumors. However, because adjuvant therapy with tamoxifen is standard throughout the world, issues of cross resistance of tamoxifen and toremifene are important considerations for the use of toremifene in recurrent breast cancer. Laboratory studies by Osborne *et al.* [280] have demonstrated that toremifene stimulated tumors can develop from MCF 7 breast cancer cells transplanted into athymic mice. Toremifene is cross resistant with tamoxifen in tamoxifen stimulated breast cancer in the laboratory [281]. Similarly, cross over clinical trials demonstrate that there is little possibility of a second response to toremifene after tamoxifen failure [282, 283].
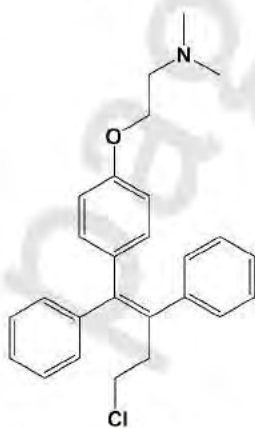


**Figure 5.15** Toremifene.

The interesting property of toremifene is the reduced liver carcinogenicity in the rat [284, 285]. Toremifene produces fewer DNA adducts than tamoxifen [284]; however, there are reports of DNA damage [286] and the drug can still act as an

estrogen like tumor promoter in the rat [197]. The lower potential to produce DNA adducts probably reflect an inability of toremifene to produce the α OH metabolite observed with tamoxifen (α OH toremifene). The chlorine of toremifene would sterically prevent α hydroxylation. Additionally, even if toremifene could be metabo lized to α OH toremifene to significant levels, rats treated intraperitoneally with α OH toremifene showed a large reduction by 39 fold in hepatic DNA adduct formation compared to tamoxifen [287]. This low level of DNA adducts generated by α OH toremifene may be due to its limited esterification and/or the poor reactivity of its sulfated and activated form α sulfoxytoremifene [287].

Issues of the incidence of endometrial cancer during toremifene therapy are controversial. Toremifene can support the growth of tamoxifen stimulated endome trial cancers in athymic mice [228], so it would not be unreasonable to predict a modest rise in endometrial cancer in patients treated long term with adjuvant toremifene. The general pharmacology of toremifene in the endometrium and uterus is the same as that of tamoxifen [288]. However, an analysis of side effects in adjuvant studies shows no increases in endometrial cancer with toremifene [289].

### 5.6.3
### Idoxifene

Idoxifene (Figure 5.16) is a metabolically stable analog of tamoxifen synthesized to avoid the toxicity reported with tamoxifen in rat liver [290 292]. Substitution of halogens at the 4 position of tamoxifen is known to reduce the antiestrogenic potency by preventing the conversion of the parent drug to 4 OH tamoxifen [157]. Addition ally, it was argued that by reduction of demethylation, liver toxicity would be reduced because increased local levels of formaldehyde would not occur [291, 292]. Unfortu nately, the increased metabolic stability also increases toxicity, since the drug cannot easily be detoxified. Idoxifene accumulates such that high parent drug levels are observed which can cause death in mice at doses that are safe for tamoxifen [281].
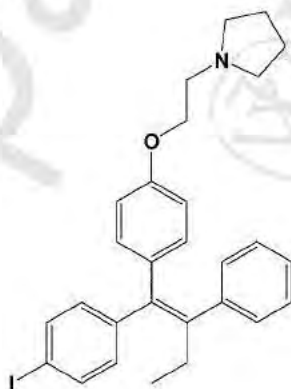


**Figure 5.16** Idoxifene.

Idoxifene inhibits the growth of carcinogen induced rat mammary tumors [293] and MCF 7 tumors grown in athymic mice [294, 295]. When compared to tamoxifen, idoxifene appears to have more antagonistic and less agonistic effects on ER in laboratory studies. Also, idoxifene has been reported to develop acquired antiestro gen resistance more slowly than tamoxifen [294]. However, there appears to be cross resistance in laboratory models of tamoxifen stimulated growth [281].

Idoxifene has been evaluated as a breast cancer treatment for postmenopausal patients [296, 297]. In one study, 321 postmenopausal patients with unknown receptor status or hormone receptor positive metastatic breast cancer were randomized to receive either tamoxifen or idoxifene as first line endocrine therapy for their advanced disease. Complete plus partial response rates were 9 and 13% for tamoxifen and idoxifene, respectively. The median time to progression was slightly higher for idoxifene (140 versus 166 days), but these differences were not statistically significant. Morbidity was similar for both groups. The authors concluded that in postmenopausal women with metastatic breast cancer idoxifene had similar efficacy and toxicity to tamoxifen [298]. However, idoxifene has not been developed further because of concerns about uterine prolapse [299]. This side effect is not seen with tamoxifen.

### 5.6.4
### Droloxifene

Droloxifene (Figure 5.17), or 3 OH tamoxifen, is a mimic of the tamoxifen metabolite 3,4 diOH tamoxifen that has weak estrogenic properties in the mouse and weak antiestrogenic actions [151, 300]. Droloxifene has antitumor activity in laboratory animals [301], and does not form DNA adducts under laboratory conditions or produce liver tumors in rats [301, 302]. Droloxifene maintains bone density in rats [303, 304], but clinical trials for the prevention of osteoporosis have not been reported. Droloxifene also reduces LDL cholesterol and lipoprotein(a) to a greater degree than conjugated estrogen in postmenopausal women [305]. However, like tamoxifen and raloxifene, droloxifene does not increase HDL cholesterol. Drolox ifene also dramatically reduces fibrinogen.
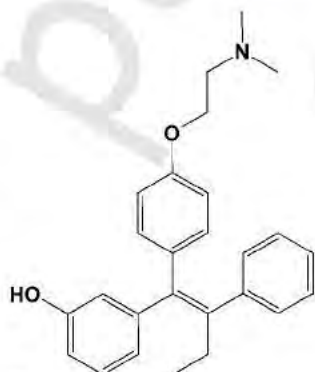


**Figure 5.17** Droloxifene.

These data lead to the extensive clinical testing of droloxifene in stage IV breast cancer [306]. In a phase III trial for treatment of ER and/or PR positive advanced breast cancer, droloxifene was found to be significantly less effective than tamoxifen overall [307]. As might be anticipated for an agent that has rapid clearance because it is rapidly conjugated by phase II metabolizing enzymes [308, 309], doses of 60 mg daily were used in its clinical trials, and may explain why droloxifene was inferior to tamoxifen. Its further clinical development has therefore been stopped.

### 5.6.5
### Ospemifene (Deaminohydroxytoremifene, FC-1271a)

Ospemifene (Figure 5.18), or deaminohydroxytoremifene, is related to metabolite Y formed by the deamination of tamoxifen [234]. Metabolite Y has a very low binding affinity for ER [234, 239] and has weak antiestrogenic properties compared with tamoxifen. Ospemifene is a known metabolite of toremifene (4 chlorotoremifene) but unlike tamoxifen, there is little carcinogenic potential in animals [310]. It is possible that the large chlorine atom on the 4 position of toremifene and ospemifene reduces α hydroxylation to the ultimate carcinogen related to α OH tamoxifen. Ospemifene has very weak estrogenic and antiestrogenic properties *in vivo* [311], but demonstrates SERM activity in bone and lowers cholesterol. The compound is proposed to be used as a preventative for osteoporosis. A phase II trial demonstrated that ospemifene decreased bone resorption markers and increased bone formation markers in postmenopausal women as well as did raloxifene with one difference; the highest dose of ospemifene evaluated (90 mg) increased the bone formation marker procollagen type I N propeptide significantly more than raloxifene [312]. Interestingly enough, unlike raloxifene, ospemifene has a strong estrogen like action in the vagina, but neither ospemifene nor raloxifene affect endometrial histology [313, 314]. Over all, the goal of developing a bone specific agent is reasonable, but the key to commercial success will be the prospective demonstration of the prevention of breast and endometrial cancer as beneficial side effects. This remains a possibility based on prevention studies completed in the laboratory [315, 316].
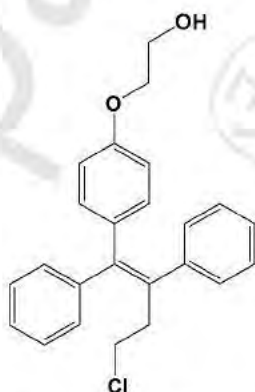


Figure 5.18 Ospemifene.

**5.6.6**

**GW5638 and GW7604**

The search for other SERMs which act on ER with a mechanism distinct from tamoxifen and raloxifene has led to the identification of GW5638 (Figure 5.19), a structural analog of tamoxifen, except GW5638 contains an acrylate side chain that replaces the dimethylaminoethoxy side chain in tamoxifen. Like tamoxifen, GW5638 is metabolized to its hydroxylated derivative, GW7604 (Figure 5.20), analogous to 4 OH tamoxifen. GW5638 behaves as an antiestrogen in breast, shows minimal uterotrophic activity in ovariectomized rats, yet protects against bone loss and decreases serum cholesterol [317, 318]. Transcriptional luciferase reporter gene studies have shown that GW5638 inhibits the agonistic activity of E2, tamoxifen and raloxifene, and derepresses the antagonist activity of the pure antiestrogen fulvestrant [318]. GW5638 inhibits the growth of E2 dependent MCF 7 breast tumors in athymic mice [319]. GW5638 is better able than raloxifene to block E2 induced growth of endometrial tumors [320]. As GW5638 downregulates ER protein le vels [321], this interesting new SERM has demonstrated non cross resistance with tamoxifen in the tamoxifen stimulated MCF 7 model in athymic mice [319, 320]. Extensive clinical trials would be appropriate if further laboratory data are obtained.
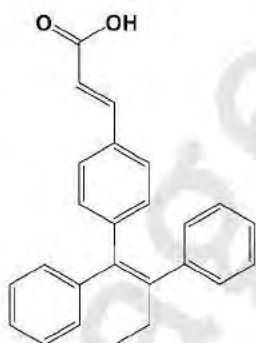


**Figure 5.19** GW5638.
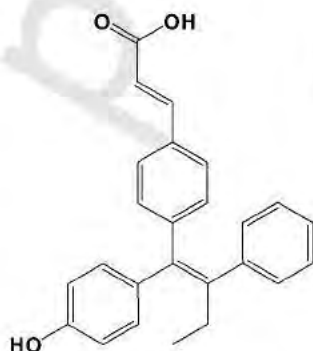


**Figure 5.20** GW7604.

GW5638 also shows promise for development as a second line agent of advanced breast cancer; in contrast to raloxifene, GW5638 effectively blocks the growth of tamoxifen resistant breast tumors in athymic mice [319, 320]. Thus, the mechanism of antitumor action of GW5638 is different from that of tamoxifen. These characteristics indicate that GW5638 could be further developed as second line therapy for the treatment of advanced breast cancer and could prove beneficial in the adjuvant and preventive settings.

GW5638 induces a unique conformational change in ER. Using phage display, synthetic peptides which interact with GW5638 bound ER were identified that did not interact with tamoxifen, raloxifene or fulvestrant bound ER [319], indicating that the conformation of GW5638 bound ER is unique relative to the other antiestrogen bound ER complexes. In MDA MB 231 cells stably transfected with wild type ER, 4 OH tamoxifen acts as an agonist, but GW7604 acts as an antagonist. However, in MDA MB 231 cells stably transfected with a mutant ER containing Asp → Tyr, GW7604 acts as an agonist [322]. Thus, Asp351 serves as a molecular switch, determining the mode by which GW5638 acts. Molecular modeling has indicated that the dimethylamino moiety of 4 OH tamoxifen weakly interacts with Asp351, but the acrylate side chain of GW7604 would be deprotonated at physiologic pH and therefore repulse Asp351 [322]. In contrast, the crystal structure of GW5638 complexed with ER [321] suggests that because the acrylate side chain of GW5638 is buried by hydrophobic residues, the acrylate side chain would be protonated instead of deprotonated, and allow formation of hydrogen bonds between it and Asp351. Noteworthy, the ER GW5638 crystal was formed at acidic pH. Also according to the crystal structure, when 4 OH tamoxifen complexes with ERα, ERα's H12 takes on the position otherwise occupied by the coactivator in a wedge formed by H3, H4 and H5. However, the acrylate side chain of GW5638 forms water mediated hydrogen bonds with Leu536 and Tyr537 located at the N terminus of H12, drawing this portion of H12 closer to the ligand. These hydrogen bonds then cause an approximately 50° difference in the orientation of H12 between the GW5638 ERα and the 4 OH tamoxifen ERα structures. This rotation of H12 leads to exposure of hydrophobic residues in H12 to the protein exterior and, hence, increases in surface hydrophobicity. E2 and fulvestrant bound to ER also increase the surface hydrophobicity of ER, which is linked to decreased protein stability. Similarly, GW5638 leads to decreased ER protein levels, likely by increasing ER surface hydrophobicity [321] and a hyperubiquitinated ER complex [251], but does not cause as much degradation of ER as does fulvestrant. A recent structure activity study of GW7604 demonstrated confirmed that the acrylate side chain was critical for the downregulation of ER levels in MCF 7 breast cancer cells [323]. In contrast, 4 OH tamoxifen does not increase ER surface hydrophobicity and hence allows for a more stable ER protein. Therefore, GW5638 and GW7604 may exert more antiestrogenic activity than tamoxifen and raloxifene.

### 5.6.7
### Lasofoxifene (CP-336156)

Lasofoxifene (Figure 5.21), also termed CP 336156, is a diaryltetrahydronaphthalene derivative [324] that has been reported to have high binding affinity for ER and have

potent activity in preserving bone density in the rat [325, 326]. Lasofoxifene also exerts potential cardioprotective effects of estrogen, but lacks estrogen's endometrial cancer risks [327]. The structure of CP336156 is reminiscent of the putative antiestrogenic metabolite of nafoxidine [328] that failed to become a breast cancer drug because of unacceptable side effects [329]. There are two diastereometric salts. CP336156 is the *l* enantiomer that has 20 times the binding affinity of the *d* enantiomer. Studies demonstrated that the *l* enantiomer had twice the bioavailability of the *d* enantiomer. The authors ascribed the difference to enantioselective glucuronidation of the *d* isomer [324]. An evaluation of CP336156 in the prevention and treatment of *N* nitroso *N* methylurea (NMU) induced rat mammary tumors showed activity similar to that of tamoxifen [330]. It is currently being evaluated in a worldwide phase III trial to determine if it can reduce the risk of vertebral fractures, breast cancer and cardiovascular disease in postmenopausal women [327].
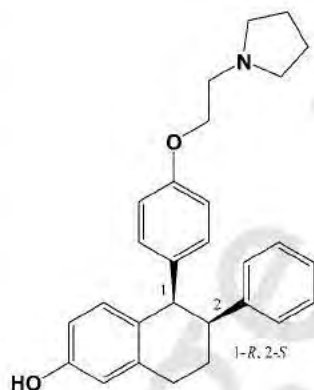


**Figure 5.21** Lasofoxifene.

### 5.6.8
### Levormeloxifene

Levormeloxifene (Figure 5.22) is the *l* enantiomer of the racemic chromane orme loxifene (centchroman), which was marketed in India as a birth control pill [331]. Levormeloxifene was intended for development of prevention of osteoporosis and coronary artery atherosclerosis. In rabbits, it lowered plasma cholesterol and reduced atherosclerosis to a similar extent as estrogen in rabbits, without estrogenic effects on uterine tissue [331]. In ovariectomized cynomolgus monkeys, levormeloxifene prevented increases in serum markers of bone turnover induced by the ovariectomy and inhibited loss of lumbar spine bone mineral density compared to placebo [332]. The beneficial effects levormeloxifene translated to the clinic [333], it decreased LDL cholesterol more than HRT without changing HDL cholesterol compared to placebo in healthy postmenopausal women. Levormeloxifene also increased bone mineral density in the spine, total hip and total body, and decreased bone turnover markers. However, unlike in rabbits, levormeloxifene increased endometrial thickness. It was

later shown in rats [334] and pigs [335] that levormeloxifene exerted estrogenic effects on the uterus. In other clinical studies in healthy postmenopausal women, beneficial effects on bone and LDL cholesterol were seen [336, 337], but in one study an addi tional estrogen like effect of levormeloxifene was also found on the hypothalamic pituitary axis since 50% reductions in FSH and LH were observed in a separate clinical study [336]. Levormeloxifene exhibited rapid absorption with slow elimination (plasma half life of approximately 1 week in postmenopausal women) [336, 338], which led to small fluctuations in steady state plasma concentration and drug accumulation. The slow elimination of levormeloxifene was consistent with the inability to determine a minimal effective dose of the drug in clinical trials and, importantly, may also help explain the noted increase in endometrial thickness associated with the compound. Endometrial safety of levormeloxifene was explored [339] by monitoring closely its effects on endometrial thickness and the serum level of an endometrial secretory protein, placental protein 14, in healthy postmenopausal women compared to raloxifene or placebo. At all doses used, levormeloxifene induced large increases in endometrial thickness and placental protein 14 compared to raloxifene. Concurrently, a large multicenter phase III study to evaluate levormelox ifene on osteoporosis was halted after 10 months because of multiple adverse gynecologic and other events, including increased endometrial thickness, enlarged uterus, uterovaginal prolapse and urinary incontinence [340, 341]. Hence, further clinical development of levormeloxifene has been stopped.
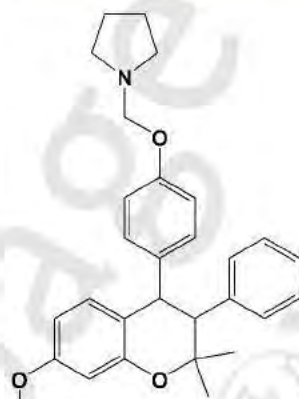


**Figure 5.22** Levormeloxifene.

### 5.6.9
### CHF 4227

CHF 4227 (Figure 5.23) is a benzopyran derivative that binds ERα and ERβ with high affinity, and exhibits an improved SERM profile. In rats, CHF 4227 inhibited estrogenic effects on uterine weight gain about 25 fold more potently than raloxifene, while also preventing ovariectomized induced bone mineral density loss and an

improved serum lipid profile comparable to the estrogen, whereas raloxifene was less effective and about 100 fold less potent [342]. Importantly, CHF 4227 also prevented 7,12 dimethylbenzanthracene (DMBA) induced mammary carcinomas [342]. A separate preclinical study confirmed the beneficial effects of CHF 4227 on bone with no uterotropic effects and also noted that lasofoxifene was less effective than CHF 4227 in the preservation of trabecular microarchitecture [343]. In a clinical trial designed to evaluate tolerability, safety and its pharmacological profile [344], CHF 4227 did not cause any increases in endometrial thickness or placental protein 14, or any vaginal bleeding. CHF 4227 decreased total and LDL cholesterol. It also decreased bone resorption markers. Like levormeloxifene, CHF 4227 showed a long elimination half life, but unlike levormeloxifene, CHF 4227 was slowly absorbed [344]. Hence, CHF 4227 shows promise as a therapeutic since it appears efficacious, was well tolerated, and seems to exhibit an improved safety profile compared to levormeloxifene.
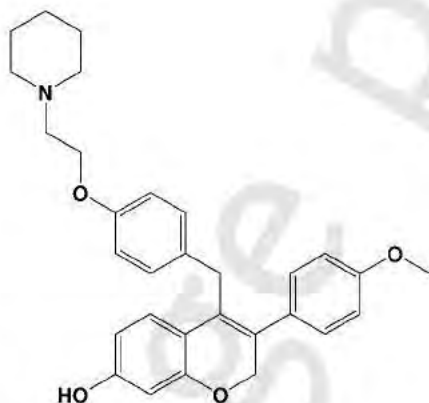


**Figure 5.23** CHF 4227.

### 5.6.10
### EM-800 and Acolbifene (EM-652, SCH57068)

EM 800 (Figure 5.24) is a chromene prodrug [324] of the active agent EM 652 (SCH57068) that is now called acolbifene (Figure 5.25). The agent is routinely drawn to show the similarity of side chain position to the pure antiestrogen fulvestrant (Figure 5.30); however, the compound is a SERM. The advantage with EM 800 and EM 652 is that they are both pure (*S*) enantiomers. Resolution of the active (*S*) enantiomer from the less active (*R*) enantiomers EM776 and EM 651 confers higher binding affinity for ER. A comparison of the potent benzopyran described by Sharma *et al.* [345] (referred to as EM312 by Gautier *et al.* [324]) with EM 652 on the proliferation of ZR 75 1 and T47D cells shows that EM 652 is 9 and 28 times more potent, respectively [324].
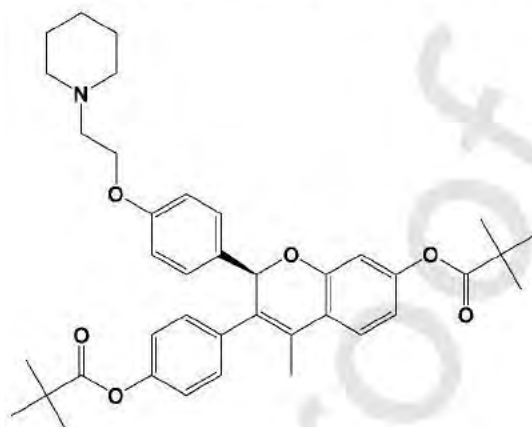
**Figure 5.24** EM 800.
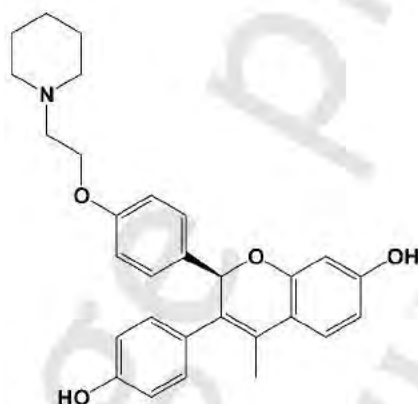


**Figure 5.25** Acolbifene.

The compound EM 800 and its active metabolite EM 652 are both orally active agents with virtually no uterotropic activity [324]. EM 800 is an active antitumor agent in the rat DMBA model [346, 347], and long term studies in the mouse show clear cut antiestrogenic activity [348] with little or no estrogenic activity compared with either tamoxifen or toremifene [349, 350]. The drug is extremely potent against breast and endometrial cancer cells in culture [351, 352] and prevents the growth of estrogen stimulated tumor xenografts in athymic mice [353]. However, unlike fulvestrant, which has an expected negative effect on bone density [354], EM 800 does not decrease bone density in the rat [346].

EM 652 is misclassified as an orally active pure antiestrogen [91, 349, 355] and as such could be tested as a second line therapy following tamoxifen failure. The antiestrogenic side chain of EM 652 would seem to be too short for optimal pure antiestrogen activity [356]. On the basis of the structural similarity of EM 652 with other benzopyrans and raloxifene analogs one would predict that EM 652 would be a SERM. A recent report demonstrates that EM 652 and raloxifene both have the

antiestrogen side chain interacting with amino acid 351 in ER [357]. The Asp → Tyr ER mutant converts both EM 652 and raloxifene to an estrogenic complex, whereas fulvestrant does not. On the basis of these data, there is potential that acolbifene's may fail as a second line therapy after drug resistance to tamoxifen develops. In a phase II study to determine the efficacy and safety of EM 800 [358], 43 postmeno pausal women with advanced breast cancer who had progression of disease while on tamoxifen were randomized to receive two different doses of EM 800. An objective response was seen in 12% of patients with one patient experiencing a complete response. Hence, acolbifene's cross resistance with tamoxifen is incomplete, yet applying this agent in first line therapy before antihormonal resistance develops would seem to be more appropriate.

### 5.6.11
### Arzoxifene (LY353381)

The benzothiophene arzoxifene (Figure 5.26) is the same molecule as raloxifene except for replacement of the ketone group with a methoxy group. The methoxy modification results in decreased metabolic elimination of arzoxifene and improved bioavailability over raloxifene [359]. Arzoxifene displays exceptionally high affinity for ER in comparison to other SERMs. *In vitro* and *in vivo* studies have demonstrated that arzoxifene exhibits a 10 fold increase in antiestrogen potency, and it does not promote uterine growth [359]. Arzoxifene is partially cross resistant with tamoxifen in models of drug resistant breast and endometrial cancer [322, 360].
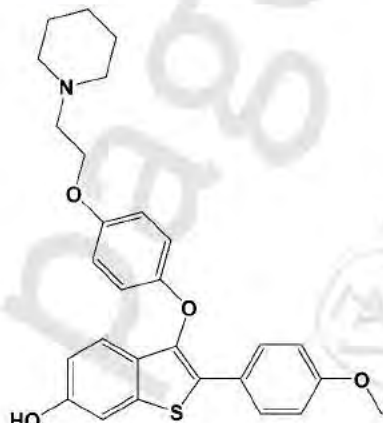


**Figure 5.26** Arzoxifene.

In phase II clinical trials of women with advanced breast [361, 362] and endome trial [363] cancer, arzoxifene proved to be marginally effective (response rates 10 30%) with minimal toxicity. However, its main role may be in the prevention of breast cancer as with raloxifene [364]. Arzoxifene has been shown to be superior to

raloxifene as a chemopreventive in rat mammary carcinogenesis [69]. Therefore, due to arzoxifene's improved pharmacokinetic profile over raloxifene, arzoxifene should be evaluated in a chemoprevention trial to determine whether it would decrease the risk of invasive breast cancer more than raloxifene.

### 5.6.12
### Bazedoxifene (TSE-424)

Structurally similar to raloxifene, bazedoxifene (TSE 424; Figure 5.27) is under development as a SERM with improved tissue selectivity for use in the prevention and treatment of osteoporosis in postmenopausal women and in combination with HRT [365]. Bazedoxifene binds to ERα with a similar affinity as that of raloxifene, blocks E2 induced growth of MCF 7 cells in culture, increases bone mineral density and compressive bone strength in ovariectomized rats, yet promotes less uterotropic effects than raloxifene in an immature rat uterine model, and when coadministered with raloxifene, reduces raloxifene induced cellular hypertrophy [366]. Endometrial effects of escalating doses of bazedoxifene have been clinically evaluated in postmenopausal women [367]. Doses of bazedoxifene from 2.5 to 20 mg/day resulted in no significant changes in endometrial thickness or amenorrhea rates compared to placebo. Interestingly, increased doses of 30 and 40 mg/day bazedoxifene were significantly associated with both decreased endometrial thickness and uterine bleeding. This apparent antagonism in the endometrium is a unique characteristic of this SERM. Bazedoxifene is currently being evaluated in international phase III clinical trials with combined enrollment goals of 9000 women for the prevention and treatment of postmenopausal osteoporosis [368]. Also like raloxifene, it may be worthwhile to evaluate bazedoxifene for use in breast cancer prevention.
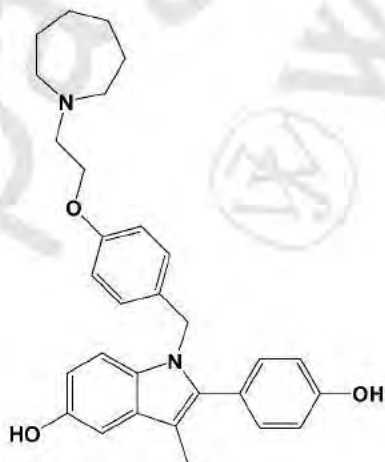


**Figure 5.27** Bazedoxifene.

## 5.6.13
## HMR 3339

Unlike the other SERMs, HMR 3339 (Figure 5.28) is a steroid. It is under clinical development for prevention of osteoporosis and coronary heart disease. In rats, HMR 3339 completely prevented bone mineral density loss and even increased it following ovariectomy. Further, HMR 3339 also increased bone mechanical strength at multiple sites following ovariectomy. HMR 3339's increases in bone mineral density and bone strength were more pronounced than raloxifene on cortical bone [369]. In healthy postmenopausal women, HMR 3339 promoted an antiatherogenic lipid profile by reducing total and LDL cholesterol, and decreased homocysteine levels, while not influencing HDL cholesterol and lipoprotein(a), whereas raloxifene showed similar effects but did not reduce homocysteine levels [370]. In a separate clinical study exploring effects of HMR 3339 on markers of coagulation and fibrinolysis in healthy postmenopausal women [371], HMR 3339 at the highest dosage (50 mg daily) reduced antithrombin, protein C, and fibrinogen compared to placebo. At the lowest dosage (2.5 mg daily), HMR 3339 showed beneficial effects on some markers of fibrinolysis by decreasing tissue type plasminogen activator, plasmin $\alpha_2$ antiplasmin complex and D dimer compared to placebo. HMR 3339 also reduced the fibrinolysis inhibitor pro carboxypeptidase U [372] and showed a dose dependent reduction in C reactive protein [373]. Additional beneficial effects of HMR 3339 on the cardiovasculature in postmenopausal women have been found by observing that it caused a dose dependent decrease in the nitric oxide synthase inhibitor asymmetric dimethylarginine, whereas raloxifene did not [374]. Hence, HMR 3339 shows promise for protection of coronary heart disease, and perhaps osteoporosis.
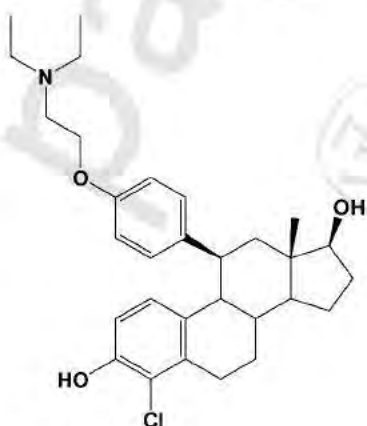


**Figure 5.28** HMR 3339.

**5.7**
**Pure Antiestrogens**

By definition, a compound that is a pure or complete antiestrogen in all laboratory tests is unlikely to be selectively active in humans. To produce antiestrogen action at all sites, pure antiestrogens have a unique mechanism of action. The compounds have no intrinsic activity by preventing the formation of a transcription complex at target genes and the ligand enhances the ability of the ER complex to be destroyed. The use of pure antiestrogens for the adjuvant treatment of breast cancer is appealing if the benefits in lives saved are not confounded by increases in osteoporosis and coronary heart disease. Although pure antiestrogens were first described by Wakeling and Bowler [375] almost 20 years ago, there is remarkably little information about adverse effects of these drugs on bones and lipids. Drug development has been slow. The concern about increased risk of osteoporosis and coronary heart disease, as well as problems with drug delivery, has encouraged the development of aromatase inhibition as an alternative strategy for 'antiestrogen action' without the endometrial complications observed with tamoxifen. Nevertheless, there is clearly a strategic role for the pure antiestrogen fulvestrant (see below) in the treatment of advanced breast cancer [376, 377] when the patient may or may not have received 5 years of adjuvant tamoxifen. Additionally, a pure antiestrogen could find a role in the adjuvant treatment of high risk (four or more lymph node positive) breast cancer. It is clear, however, that the application of a pure antiestrogen will compete with the established methods of estrogen deprivation with AIs (postmenopausal) or LH releasing hormone super agonists (premenopausal) which would cause a medical oophorectomy [378, 379].

**5.7.1**
**ICI 164,384 and Fulvestrant (ICI 182,780; Faslodex®)**

The first generation pure antiestrogen ICI 164,384 (Figure 5.29) is a 7α substituted derivative of E2 that has no detectable estrogen like properties *in vivo* or *in vitro* [375, 380]. The compound was identified in a search for drugs that do not possess the estrogen like effects of tamoxifen and that would, as a result, be more effective antitumor agents.
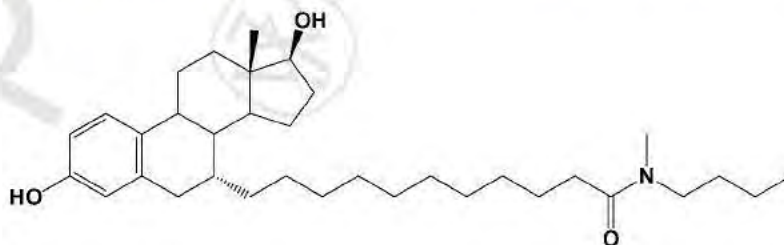


**Figure 5.29** ICI 164,384.

Originally, the inspiration to substitute E2 at the 7α position came from the observation that ER could be purified on resin columns derivatized with E2 through a

7α carbon chain linker of 10 atoms [381]. Structure activity relationships at the 7 carbon position clearly demonstrated that 7β substitutions are ineffective at producing antiestrogenic activity and the length of the carbon chain determines optimal activity for 7α substitutions [356].

Fulvestrant (Figure 5.30) is a second generation pure antiestrogen that is more potent than ICI 164,384 [382] and has fluorine atoms at the terminus of the 7α side chain to retard metabolism to estrogen.
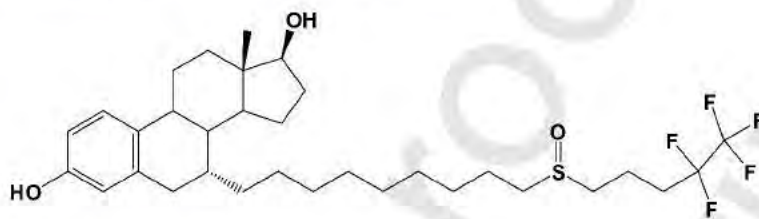


**Figure 5.30** Fulvestrant.

Although the pure antiestrogen ER complex exerts no agonist activity there is another dimension to the mechanism of the pure antiestrogens that appears to be unique. Initially, it was believed that pure antiestrogens prevent the dimerizations of receptor complexes, thereby preventing the binding to EREs [383]. Clearly, if receptor complexes do not bind to any ERE, then no gene can be activated and the compound would be 'a pure antiestrogen'. However, investigators [383, 384] have subsequently demonstrated that the pure antiestrogen ER complex does bind to EREs, but both AF 1 and AF 2 are inactivated. What appears to be unique about pure antiestrogens is the observation that they provoke the rapid destruction of ER in breast cancer cells in culture [385], mouse uterus [386], and breast tumors *in situ* [387]. ER is synthesized in the cytoplasm and transported to the nucleus where it functions as a transcription factor. Once a pure antiestrogen binds to the newly synthesized receptor in the cytoplasm, transport of the ER complex to the nucleus is impaired [388]. Further, fulvestrant binding to ER induces increased surface hydrophobicity [321] and an abnormal conformation that leads to accelerated ubiquitination and shuttling of ER to the proteasome for degradation [389]. Although normal target cells could be affected in the long term, the loss of ER in a breast tumor cell will immediately prevent cell survival and result in tumor regression.

Crystal structures of ER complexed with pure antiestrogens would provide a wealth of mechanistic information, but generating these crystals have proved challenging. However, the crystal structure of the rat ERβ LBD and ICI 164,384 has been resolved, but due to internal disorder, the crystal had required treatment with *p* chloromercuribenzenesulfonic acid [390]. This results in a distorted homodimer structure. There are several similarities and differences of the crystal structure of ICI 164,384 with ERβ when compared to that observed with raloxifene in ERα or ERβ [42, 45]. The bulky *para* substituted phenyl side chains of raloxifene and 4 OH tamoxifen occupy a narrow channel in ER, pushing H12 aside to silence AF 2. ICI 164,384 adopts a similar binding mode by flipping 180° about its longest

hydroxyl to hydroxyl axis (Figure 5.31). In this conformation, the 7α substituted side chain can exit the binding cavity. This molecular solution has been suggested previously to describe the antiestrogenic activity of the 11β substituted estrogen RU 39,411 and ICI 164,384 [391]. The unique aspect of the X ray crystallography is the finding that the long hydrophobic side chain prevents the binding of H12 to the surface of the LBD. Although the side chain exits the binding pocket in a manner identical to that observed with raloxifene, the side chain is bent by 90° at its fifth carbon and binds against the indole face of Trp290. The antiestrogenic side chain is 6 Å longer than the side chain of raloxifene so that it extends deep into the groove between H3 and H5. As a result, H12 cannot dock on the surface of the LBD [390]. This unique structure presumably results in the premature destruction of the complex by the proteasome.
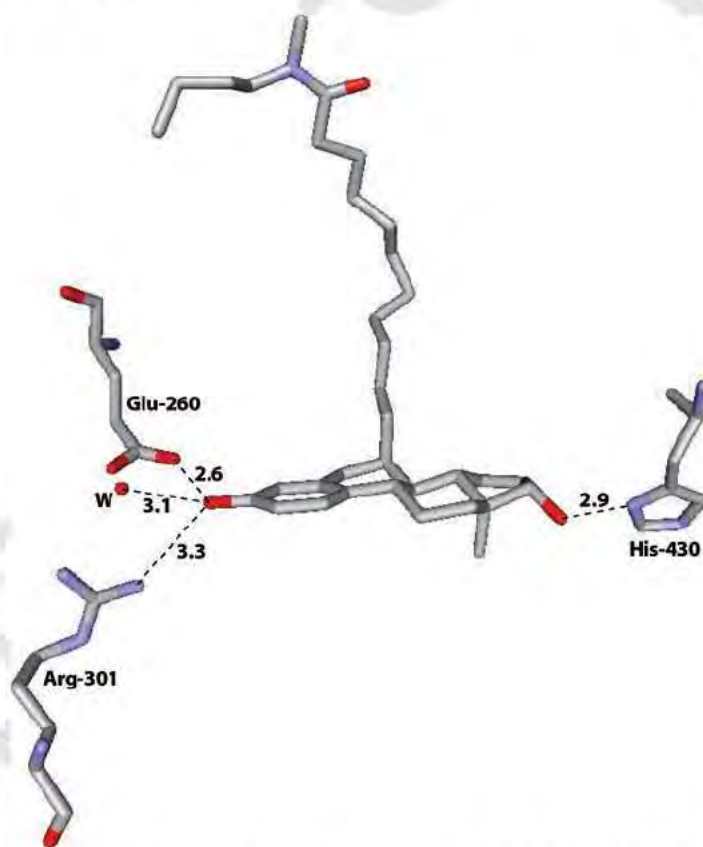


**Figure 5.31** Hydrogen bond interactions between ICI 164,384 and ERβ. Hydrogen bond intermolecular interactions of 4 OH tamoxifen cocomplexed with rat ERβ LBD using the X ray crystallographic structure 1HJ1 at 2.3 Å resolution [390] are shown. The conformation was visualized using 3D Mol Viewer as in Figure 5.5. Hydrogen bonds are indicated by dashed lines and their lengths in angstroms as determined by 3D Mol Viewer. A highly ordered water molecule stabilized by a hydrogen bond network is indicated (W). Carbon atoms are shown in gray, oxygen atoms in red and nitrogen atoms in blue.

Fulvestrant has been compared to tamoxifen for the treatment of advanced breast cancer in postmenopausal women. In a study of 587 patients, there was no significant difference between fulvestrant and tamoxifen at a median follow up of 14.5 months for the primary endpoint of time to progression (median 6.8 versus 8.3 months, respectively); however, the tamoxifen group showed slightly better results. Hence, fulvestrant displayed similar efficacy to tamoxifen and was well tolerated [392].

The steroidal pure antiestrogens ICI 164,384 and fulvestrant are not cross resistant with tamoxifen in laboratory models of tamoxifen stimulated breast [227, 393], and endometrial cancer [132] grown in athymic mice. However, drug resistance to fulvestrant does occur in cell culture [394, 395]. Fulvestrant is active as a second line agent, following tamoxifen failure for the treatment of advanced breast can cer [376, 377]. As shown by two large multicenter, randomized trials showed fulvestrant (250 mg, once monthly via intramuscular injection) to be as effective as the AI anastrozole for the treatment of postmenopausal women with hormone receptor positive advanced breast cancer progressing on prior endocrine therapy. In one study, 451 patients were randomized to receive fulvestrant or anastrozole. At a median follow up time of 14.4 months, fulvestrant was as effective as anastrozole with a median time to progression of 5.5 months for fulvestrant and 5.1 months for anastrozole, and objective response rates of 20.7 and 15.7%, respectively. Both treatments were well tolerated [376]. In the other study, 400 advanced breast cancer patients whose disease had progressed on prior endocrine treatment were random ized to receive fulvestrant or anastrozole. Fulvestrant was as effective as anastrozole in terms of time to progression (median 5.4 months for fulvestrant versus 3.4 months for anastrozole) and objective response rates were 17.5% with both groups. Both treatments were well tolerated [377]. Hence, the drug is approved in the US and Great Britain as a second line therapy for advanced breast cancer.

The destruction of ER and the removal of ER signal transduction by fulvestrant in antihormonal resistant breast cancer were anticipated to be very effective, given its impressive results in the laboratory. However, response rates seen in these clinical trials have not been as good as initially anticipated, with only two in five tamoxifen resistant patients responding to fulvestrant. One of the reasons for this may be the fact that plasma concentration levels achieved in clinical studies with the usual dose of 250 mg by intramuscular injection given once a month are significantly lower than those achieved in the media of most cell culture studies (100  1000 nM). After a single intramuscular dose of long acting fulvestrant the mean minimum and maximum plasma concentrations achieved are 2.6 (4.3) and 8.2 ng/ml (13.5 nM), respectively (reviewed in Ref. [396]), and although a 2  to 3 fold accumulation with continuous dosing of fulvestrant has been observed, the mean concentrations after 6 months of the same dosing are only 6.1 ng/ml (10.05 nM) [396], which still remains significantly lower than that used in cell culture studies.

Although fulvestrant has not proven to be better than other therapies, it clearly represents an additional treatment option for women with breast cancer whose disease fails to respond to other therapies. Currently, its use as a third line therapy results in a 28% rate of stable disease with a partial response in this group of patients. In addition, its high tolerability profile and novel mode of action offer the potential for

its use in combination with other therapeutic regimens. Indeed, a number of trials are currently evaluating the role of fulvestrant in association with other agents such as AIs, trastuzumab (HER2 targeted antibody), lapatinib (dual EGFR and HER2 tyrosine kinase inhibitor) and other agents [397, 398].

### 5.7.2
### Additional Pure Antiestrogens

#### 5.7.2.1 ZK-703 and ZK-253

Fulvestrant has proven to be as effective as the AI anastrozole in patients who relapsed during treatment with tamoxifen [376]; however, as described earlier it has very low bioavailability. To avoid this problem, the novel pure antiestrogenic compounds ZK 703 (Figure 5.32) and ZK 253 (Figure 5.33) have been developed [399]. Both are administered subcutaneously; however, ZK 253, a structurally optimized form of ZK 703, is orally bioavailable and retains its antiproliferative activity when administered via this route in *in vivo* xenograft breast cancer models. In these models, both ZK 703 (subcutaneous administration) and ZK 253 (oral administration) inhibited tumor growth better than either tamoxifen or fulvestrant. In MCF 7 xenograft tumors, subcutaneous ZK 703 led to very low levels of ER protein compared to controls. In rats, ZK 703 has shown greater oral bioavailability than in mice; hence, antitumor activity of ZK 703 and ZK 253 were evaluated in the DMBA and NMU induced rat mammary tumor models via oral administration [399]. ZK 703 and ZK 253 caused a nearly complete (greater than 80%) inhibition of DMBA induced tumor growth and ZK 703 caused a 75% inhibition of NMU induced tumor growth. Importantly, ZK 703 and ZK 253 still effectively inhibited growth of tamoxifen resistant MCF 7 xenograft tumors, whereas fulvestrant was only moderately effective. Also, ZK 703 and ZK 253 led to lower ER protein levels than fulvestrant in this model. Thus, tamoxifen resistant MCF 7 xenograft tumors are not cross resistant to ZK 703 and ZK 253 [399]. These preclinical studies suggest that these compounds, similar to fulvestrant, may have a role in the treatment of breast cancer. The fact that ZK 253 appears to be active after oral administration may prove very important when these compounds are compared with fulvestrant. Clinical studies to determine the safety and efficacy of these compounds in humans are warranted.
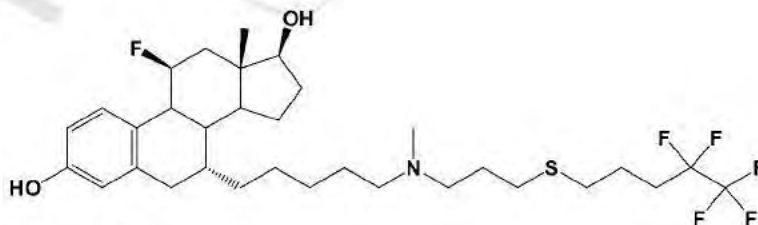


**Figure 5.32** ZK 703.
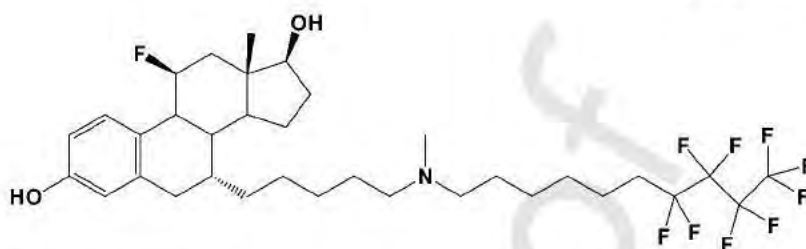
**Figure 5.33** ZK 253.

### 5.7.2.2 RU 58668

The discovery of ICI 164,384 and fulvestrant stimulated a search for other potential agents. The compound RU 58,668 (Figure 5.34) is substituted at the 11β position with a side chain of comparable length and physical chemistry as that used for fulves trant [400, 401]. RU 58668 causes a protein synthesis dependent paralysis of ER in the particulate fraction of the cytoplasm that depends entirely on an intact LBD [402]. Indeed, the authors [402] suggest that antiestrogens that block ER nuclear localiza tion will behave as pure antiestrogens *in vivo*. It has been reported to exert improved antiproliferative activities relative to fulvestrant in *in vitro* [400] and *in vivo* MCF 7 models [401]. RU 58668 also blocks E2 induced increases in uterine weights without any uterotrophic effects by itself [400]. Like fulvestrant, RU 58668 leads to greatly enhanced proteasome mediated degradation of ER [403, 404]. Hence, RU 58668 represents an alternative for pure antiestrogen therapy of breast cancer.
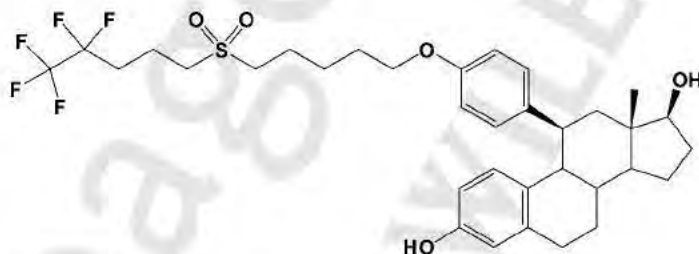


**Figure 5.34** RU 58668.

### 5.7.2.3 TAS-108

TAS 108 (SR 16234; Figure 5.35) is a steroidal compound that acts as a high affinity pure antagonist of ERα. Its mechanisms of action are different than SERMs and fulvestrant. TAS 108 recruits corepressors to ERα without affecting AF 1 activity or DNA binding [405]. Unlike the SERMs 4 OH tamoxifen and raloxifene, which activate transcription of the Asp351 to Tyr mutant of ERα (ERα Asp → Tyr) isolated from a tamoxifen resistant xenograft MCF 7 tumor [245, 249, 406], TAS 108 fails to activate transcription mediated by this ERα mutant [405]. Unlike fulvestrant, TAS 108 does show partial agonistic activity for ERβ; TAS 108 promotes recruitment of the coactivator SRC 2 to the AF 2 of ERβ [407]. This partial ERβ agonism may explain why

this drug does not cause increased loss of bone density. However, TAS 108 shows only a minimal uterotrophic effect in ovariectomized rats [407] and no uterotrophic effects in humans by trans vaginal ultrasound evaluation [408]. Preclinical studies have shown that this agent exerts antitumor effects in DMBA induced rat mammary tumors and in xenograft MCF 7 breast tumors [407]. In contrast to fulvestrant, which is administered clinically by intramuscular injection, TAS 108 is administered orally. In a phase I study, TAS 108 was administered at 40 mg/day, and dose escalation to 160 mg/day was well tolerated, showing only grade 1 2 toxicities (nausea, vomiting, hot flashes, headache, weakness and fatigue) and no maximum tolerated dose. A circulating mean $C_{max}$ of 2.8 21.0 ng/ml (5.5 41.5 nM) was achieved. Moreover, evidence of antitumor activity was observed in this phase I study [408]. Currently, a phase II study is evaluating this drug and phase III studies are being planned [409].
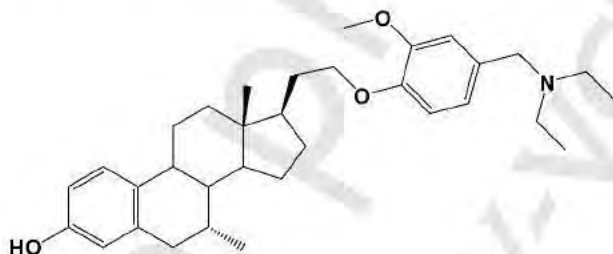


**Figure 5.35** TAS 108.

## 5.8
## Conclusions

Over the past 30 years, endocrine therapy has been proven to be the most advanta geous targeted therapy for ER positive breast cancer while sparing the patient from the debilitating toxicities of chemotherapy. Five years of adjuvant tamoxifen therapy for all stages of ER positive breast cancer, node positive and negative, confers a clear cut survival advantage. Tamoxifen is also used as a preventive in both pre and postmenopausal women at high risk for breast cancer. Tamoxifen has clearly been successful and serves as the prototype for the development of newer SERMs such as raloxifene that lack tamoxifen's undesirable utertrophic side effect. Numerous SERMs are currently under development not only for use in breast cancer, but also for use in osteoporosis and coronary heart disease. The pure antiestrogen fulvestrant has now been established as a second line therapy to inhibit the growth of breast tumors after tamoxifen failure by targeting ER for ubiquitin mediated degradation. New pure antiestrogens are under development which exhibit improved oral bioavailability over fulvestrant. A possible mechanism of antihormonal resistance includes cross talk among growth factor receptor signaling, the ER and a change in the balance of coactivators to corepressors recruited to ER. The next challenge is to

build on the success of antihormonal therapy and identify further targets to enhance antitumor activity.

## Acknowledgments

## References

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# Practical progress in the chemoprevention of breast cancer with selective estrogen receptor modulators (SERMs)

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**Introduction:**

The known link between estrogen and breast cancer suggested an application for non-steroidal antiestrogens as potential treatments for breast cancer. The majority of the compounds selected for evaluation were unsuccessful, but one compound ICI 46,474 an antiestrogenic, antifertility agent in the rat (Harper and Walpole 1967), was noted to be as effective as high dose estrogen or androgen therapy but with fewer side effects (Cole, Jones et al. 1971). The approval of tamoxifen (ICI 46,474) as an antiestrogen to treat breast cancer opened the door for a rigorous evaluation of the pharmacology of antiestrogens that ultimately led to the recognition of the concept of selective estrogen receptor (ER) modulation. The practical applications of the selective estrogen receptor modulators (SERMs) has facilitated the clinical goal of chemoprevention (Jordan 2003) and the development of raloxifene, the first multifunctional medicine.

The first evidence to show that tamoxifen acts as a reversible antiestrogen in breast cancer was noted during i*n vitro* studies utilizing MCF-7 cells (Lippman and Bolan 1975). The conclusion that tamoxifen reversibly interfered with the trophic effects of estrogen was based on three lines of evidence: a) that the inhibition of cell growth is reversible by addition of estradiol; b) tamoxifen had no effect in cell lines unresponsive to estradiol and c) tamoxifen is capable of binding to the ER (Jordan and Koerner 1975; Lippman and Bolan 1975). The antiestrogenic and antihormonal properties of tamoxifen were also demonstrated *in vivo*. Tamoxifen inhibited induction and growth of 7, 12-dimethyl benz(a)anthracene (DMBA) induced tumors in rats (Jordan 1976; Jordan and Dowse 1976; Jordon and Jaspan 1976). In addition to research that demonstrated the antiestrogenic properties of tamoxifen, further studies illustrated the unusual pharmacology of the drug; it was antiestrogenic in some species but estrogenic in others.

Tamoxifen is antiestrogenic in *Xenopus laevis* (Riegel, Jordan et al. 1986) and the chick oviduct (Sutherland, Mester et al. 1977), estrogenic in dogs (Furr and Jordan 1984) and yet both estrogenic and antiestrogenic in rats (Harper and Walpole 1967; Jordan, Dix et al. 1977), mice (Harper and Walpole 1966; Jordan, Rowsby et al. 1978) and humans (Furr and Jordan 1984). The dichotomy of tamoxifen's actions was initially attributed to species specific differences in metabolism. However, no differences in drug metabolites among various species were found (Jordan and Robinson 1987). Thus, the new, emerging concept to be developed was selective tissue targeting of the specific actions of the nonsteroidal antiestrogens.

### The recognition of selective ER modulation

An understanding of the estrogen like pharmacology of tamoxifen in the mouse was crucial to developing the idea of tissue selective ER modulation. The implantation of MCF-7 breast cancer cells into athymic mice has been exploited as a model of estrogen-stimulated breast cancer growth (Osborne, Hobbs et al. 1985). Despite the fact that tamoxifen is estrogenic in the mouse and causes increases in uterine wet weight (Terenius 1971), tamoxifen did not enhance the growth of MCF-7 cells in athymic mice (Jordan and Robinson 1987). Therefore, the target tissue rather then the host is crucial for selective ER modulation.

The concept that tissues and not species were differentially stimulated or inhibited by tamoxifen was further clarified by the findings that ER positive tumors from breast (MCF-7) and endometrial (EnCa101) origins behaved differently when implanted in the same athymic mouse despite the production of same drug metabolites (Gottardis, Robinson et al. 1988). The estrogen stimulated growth of ER positive tumor was inhibited by tamoxifen, while the endometrial tumor was stimulated by tamoxifen (Gottardis, Robinson et al. 1988). These findings further

established that metabolism does not play a role in the species specific differences of tamoxifen action, and that tamoxifen exhibits a tissue specific pharmacology.

In parallel laboratory studies tamoxifen and raloxifene (originally known as LY 156,758 or keoxifene) (Clemens, Bennett et al. 1983) prevented the development of estrogen dependent N-nitrosomethylurea (NMU)-induced mammary carcinoma (Gottardis and Jordan 1987) and maintained bone density in ovariectomized rats (Jordan, Phelps et al. 1987). While both drugs exhibited similar effects in maintaining bone density (Jordan, Phelps et al. 1987), raloxifene was less effective then tamoxifen in preventing tumor appearance at the same dose (Gottardis and Jordan 1987). Tamoxifen, in contrast to raloxifene, increased the uterine wet weights of ovariectomized rats (Jordan, Phelps et al. 1987). Most importantly, the fact that both antiestrogens delayed tumor formation and maintained bone density in the ovariectomized rat models indicated that these observations were a drug class effect.

Subsequent animal studies compared the effects of raloxifene treatment to those of ethynyl estradiol (Black, Sato et al. 1994). Raloxifene blocked decreases in bone mineral density (BMD) and had hypocholesteremic effects in rats that were almost identical to the effects of ethynyl estradiol and research previously reported for tamoxifen 15 years earlier (Harper and Walpole 1967). There were no differences in triglyceride levels between the raloxifene treated and ethynyl estradiol treated animals as compared to ovariectomized controls. Most importantly, raloxifene did not exhibit any significant effects on the uterus. Uterine wet weights of raloxifene treated animals were slightly higher than the ovariectomized controls, while the ethynyl estradiol treated animals had substantially higher uterine wet weights than the ovariectomized controls. Additional uterine parameters considered were: epithelial height, myometrial thickness, stromal expansion and stromal eosinophilia. The raloxifene treated animals exhibited no differences

when compared to the ovariectomized controls in all parameters considered. The ethynyl estradiol animals exhibited similar profiles to the intact controls and were statistically different from the ovariectomized controls.

The laboratory recognition of selective ER modulation was immediately translated to clinical advances, first to improve the safety of women treated by tamoxifen adjuvant therapy for node positive and node negative breast cancer, and subsequently, to introduce a new approach to the prevention of breast cancer by the development of drugs called SERMs. However, translational research does not follow a straight path and potentially good ideas with encouraging preliminary findings do not necessarily lead to improvements in health care. Billions of dollars have been invested in the development of the SERM concept, but clinical practice has not fulfilled the promise in its entirety. As a result, we have chosen to describe the twists and turns of the SERM story in some detail to illustrate how difficult and complicated it is to achieve success in therapeutics. The lesson learned is that the tantalizing clues that accumulate to indicate the advances in therapeutics are either possible or doomed once the evidence from prospective clinical trials are published.

**Tamoxifen and endometrial cancer**

The benefits of long term tamoxifen therapy had to be carefully examined in light of the laboratory findings (Gottardis, Robinson et al. 1988) that tamoxifen may be associated with increased incidence of endometrial cancer (Hardell 1988; Jordan 1988; Fornander, Rutqvist et al. 1989). Increases in endometrial cancer rates associated with tamoxifen therapy (Stewart HJ and GM 1989; Neven, De Muylder et al. 1994) were not found in all studies and the issue was further obscured by small sample sizes, lack of data collection or usage of higher doses of tamoxifen (40

mg daily) (Fornander, Rutqvist et al. 1989). The issue of dosage was resolved in the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 trial that determined tamoxifen benefits and reduction in the incidence of breast cancer recurrence, contralateral breast cancer and mortality at the lower, 20 mg daily dose (Fisher, Costantino et al. 1989). Furthermore, the B-14 trial contained a placebo arm that allowed for assessment of the rates of secondary cancers with emphasis on endometrial cancer. Subsequent analysis of secondary cancers, other then endometrial cancer, during the B-14 study indicated that there were no statistical differences in the rates of secondary cancers between the placebo and tamoxifen treated groups. Focus on the endometrial cancer rate of the patient population and subsequent analysis determined that the annual rate for the placebo group was 0.2 patients per 1000 and for the randomized tamoxifen treated group the annual rate was 1.6 patients per 1000. Overall analysis of all endometrial cancers observed in the study established that the vast majority of endometrial cancers occurred in postmenopausal women. Most importantly, the study found that when all categories of events were considered and combined, there was an overwhelming net benefit from tamoxifen treatment.

Meta analysis of all randomized, placebo controlled, adjuvant tamoxifen trials started before 1990 demonstrated significant tamoxifen benefits in recurrence, contralateral breast cancer and mortality (1998). Of the 55 trials in the meta analysis, 14 had a duration of less than one year, 32 trials had a 2 year duration and 9 had a 3 or more years duration (median 5 years). The analysis of recurrence as a first event and mortality indicated a highly statistically significant benefit with tamoxifen treatment. More importantly, breakdown of the trials by duration indicated that risk reduction may be dependent on the length of tamoxifen therapy and individuals that underwent longer duration of therapy received larger benefits. Additional

breakdown of the study population based on ER status indicated a significant benefit to ER positive individuals, which was most prominent in the 5 year treatment trials. Tamoxifen did not benefit individuals with ER negative tumors. The benefit of tamoxifen also applied to both node negative and node positive individuals and in both pre and postmenopusal women, regardless of age. The meta analysis also established that tamoxifen treatments increased the risk of endometrial cancer by approximately two fold, which translated in approximately four fold increase in the risk of endometrial cancer during the duration of a 5 year trial.

### The clinical demontration of selective ER modulation

Scientific principles for the effective applications of tamoxifen as a targeted adjuvant therapy (Jordan, Dix et al. 1979; Jordan and Allen 1980) translated from the laboratory to clinical practice between the mid 1970s and the early 1990s. The targeting of long term adjuvant tamoxifen therapy to patients with ER positive breast cancers was shown to enhance survivorship (1998) and contribute to falling national death rates from breast cancer (2005). However, concerns were raised during the 1980s that the strategy of long term tamoxifen treatment could result in toxicities related to the antiestrogenic effects of the drug. This debate initiated an interest in the clinical pharmacology of tamoxifen.

The effects of tamoxifen not related to breast cancer were first and specifically addressed in the Wisconsin Tamoxifen Study (Love, Newcomb et al. 1990). The study included postmenopausal women with breast cancer and histologically negative axillary lymph nodes, with a two year follow up. The primary focus of investigations was the effects of tamoxifen on plasma levels of lipids, lipoproteins, and coagulation proteins, changes in bone density and symptomatic effects. Within 3 months of treatment the tamoxifen treated group had statistically

significant decreases in total cholesterol as compared to placebo, and more importantly, this decrease was persistent throughout all observed time points (Love, Wiebe et al. 1991). The mean decrease in total cholesterol from baseline was approximately 12%. Initial results indicated decreases of high density lipoprotein (HDL) levels in the tamoxifen group, which were statistically significant at the 12 month time point. The HDL cholesterol level reduction between the two groups was not observed at the 18 and 24 month time point. Triglyceride levels were modestly increased in the tamoxifen treated group and continued to rise at the 18 and 24 months. With the exception of the 6 month time point, the increase in triglycerides was a statistically significant finding for all time points. The low density lipoprotein (LDL) cholesterol levels decreased within the first 3 months of tamoxifen treatment and were significantly reduced for the 24 month period compared to placebo (Love, Newcomb et al. 1990; Love, Wiebe et al. 1991). Assessment of side effects associated with tamoxifen treatment indicated that tamoxifen was a well tolerated agent, yet a significant number of patients developed chronic moderate to chronic severe vasomotor symptoms and/or mild gynecological symptoms (Love, Cameron et al. 1991). During the two year study, the radius BMD in the tamoxifen treated group decreased by 0.88% per year and the lumbar spine BMD increased 0.61% per year compared to baseline (Love, Mazess et al. 1992). In the placebo group, the BMD of the radius decreased by 1.29% per year and in the spine the BMD decreased by 1.00% per year, compared to base line. Comparison of the tamoxifen treated group to the placebo group indicated statistically significant differences for lumbar spine BMD but not for the radius BMD between the groups. It is important to note that the Wisconsin Tamoxifen Study included both pre and postmenopausal women. Analysis of the BMDs based on menopausal status at time of breast cancer diagnosis indicated that lumbar spine BMD increased 1.00% per year in the tamoxifen treated group of women who were post-

menopausal at time of diagnosis. There were no differences between the groups in osteocalcin levels, parathyroid hormone and 1, 25-dihydroxyvitamin D. However, after 12 months there was a significant decrease in serum alkaline phosphatase levels in the tamoxifen treated group compared to baseline and placebo groups.

The Wisconsin Tamoxifen Study indicated that toxicologically, tamoxifen is a well tolerated agent with positive effects on BMD and potentially beneficial effects on overall lipid levels. Moreover, the study indicated that tamoxifen could potentially be used in breast cancer patients for stabilization of bone mass, particularly in women where estrogen and bisphosphonates are contraindicated.

The translation of laboratory observations that tamoxifen maintains bone density in ovariectomized rats (Jordan, Phelps et al. 1987) into the clinic enhanced the possibility that the SERMs could become a novel drug group to aid postmenopausal women health.

**Chemoprevention with Tamoxifen**

Laboratory (Jordan, Naylor et al. 1980; Jordan 1981) and human epidemiological evidence (Miller and Bulbrook 1980) supporting the hypothesis that estrogens are involved in breast cancer progression raised the possibility that endocrine intervention could prevent breast cancer development. Tamoxifen was the only candidate available to directly advance the strategy of decreasing breast cancer incidence in high risk populations. However, a novel approach was also proposed to avoid many of the side effects noted with tamoxifen by developing the SERMs as multifunctional medicine. An indirect plan for breast cancer chemoprevention as a public health initiative was first described at the First International Chemoprevention Meeting in New York in 1987:

The majority of breast cancers occur unexpectedly and from unknown origin. Great efforts are being focused on the identification of a population of high-risk women to test "chemopreventive" agents. But, are resources used less then optimally? An alternative would be to seize on the developing clues provided by an extensive clinical investigation of available antiestrogens. Could analogues be developed to treat osteoporosis or even retard the development of atherosclerosis? If this proved to be true, them majority of women in general would be treated for these conditions as soon as menopause occurred. Should the agent also retain antibreast tumor actions, then it might be expected to act as a chemosupressive on all developing breast cancers if these have an evolution from hormone-dependent disease to hormone-independent disease. A bold commitment to drug discovery and clinical pharmacology will potentially place us in a key position to prevent the development of breast cancer by the end of this century. (Jordan 1988)

This proposal was subsequently refined and presented at the AACR meeting in San Francisco in 1989. The proposal stated:

We have obtained valuable clinical information about this group of drugs that can be applied to other disease states. Research does not travel in straight lines and observations in one field are major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids, so apparently, derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high-risk group to prevent breast cancer (Lerner and Jordan 1990).

Tamoxifen, a selective antiestrogen proven to delay the relapse and prolong survival (1987), was an ideal direct chemopreventive candidate due to ease of administration and low acute toxicity, which in turn indicated good long term compliance.

An overview of the characteristics of four major tamoxifen chemoprevention trials is shown in Table 1. Preliminary studies (Powles, Hardy et al. 1989; Powles, Jones et al. 1994) established that patient's medication compliance was high and similar in both the tamoxifen and the placebo groups. Most commonly associated problems with tamoxifen treatment were hot flashes which occurred in 34% of the women in the tamoxifen group and in 20% of the women

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in the placebo group. The most significant differences in hot flashes, among the groups, were between the tamoxifen and the placebo groups of postmenopausal women. Menopausal women had similar incidences of hot flashes regardless of treatment.

Overall, tamoxifen was a well tolerated agent with low acute toxicity. Even though hot flashes occurred more frequently in women on tamoxifen, the events were mild. Observed changes in lipid levels of tamoxifen treated patients indicated the potentially positive effects of tamoxifen on overall cardiovascular health. Changes in clotting factors accompanied by decreases in the fibrinogen/ anti-thrombin ratio indicated a potential decrease in risk of thrombosis.

It is very important to note that early studies such as the Royal Marsden study (Powles, Eeles et al. 1998) and the Italian randomized trials (Veronesi, Maisonneuve et al. 1998), did not detect reduction of breast cancer risk associated with tamoxifen treatment. Both studies appeared to use large patient populations but the current consensus is that the populations were too small for practical purposes. The Royal Marsden study included 2,462 pre and postmenopausal women, while the Italian trials included 5,408, pre and postmenopausal, hysterectomized women at normal risk. The Italian trials (Veronesi, Maisonneuve et al. 1998) noted one crucial observation. Women who received HRT and tamoxifen had significantly lower incidence of breast cancer compared to women in the placebo group who received HRT (Veronesi, Maisonneuve et al. 2003).

The International Breast Cancer Intervention Study (IBIS-I) (Cuzick, Forbes et al. 2002) determined a 32% decrease in the rate of breast cancer between the tamoxifen and the placebo treated groups. The decrease was significant for both invasive and non invasive cancers. Even though the tamoxifen treated group had an approximately two fold excess of endometrial cancers

as compared to placebo, the finding was statistically insignificant. There were no differences in the rates of other cancers between the two groups. Moreover, the rate of venous thromboembolic events was 2.5 times higher in the tamoxifen group. A vast majority of these events (42%) occurred within 3 months of a major surgery or after prolonged immobility. Higher numbers of spontaneous thromboembolic events were also observed in the tamoxifen group as compared to placebo, but these findings were not statistically significant. In contrast to the Italian trials, in the IBIS-I trial demonstrated no differences between the tamoxifen and placebo treated individuals receiving HRT.

The primary goal of the NSABP P-1 study (Fisher, Costantino et al. 1998) was to determine whether five years of tamoxifen administration prevented invasive breast cancer in high risk women. Secondary aims included determining incidence of myocardial infarctions (fatal and non fatal) and the potential reduction of bone fractures. The NSABP P-1 trial found a highly statistically significant decrease in the number of invasive and non invasive breast cancers in the tamoxifen treated group compared to placebo. The overall risk for invasive breast cancers in the tamoxifen treated group was reduced by 49%. There was a 69% decrease in the annual rate of ER positive cancers in the tamoxifen treated group. The rates of ER negative breast cancers remained similar in both the tamoxifen and placebo treated groups. The tamoxifen treated patients had a 2.53 times greater risk of endometrial cancer than the placebo treated individuals. No differences in the rates of other invasive carcinomas were observed between the tamoxifen and the placebo groups. In regards to the secondary end points there were no differences in the number and severity of ischemic events between the two groups. The protocol defined fractures of the hip and radius as primary fracture events. Fractures of the spine were added soon after initiation of the study. Fewer osteoporotic fracture events (hip, spine and lower radius) occurred

in women who received tamoxifen than in those who received placebo. There was an increase in the overall reduction in women over 50 years of age. The incidence of stroke was increased in the tamoxifen treated group as was the incidence of thromboembolic events. There were no significant quality of life differences between the groups except for hot flashes and vaginal discharges.

It is important to note that the independent data monitoring committee of the NSABP P-1 trial, six months before the publication of the study, determined that the primary goal of the trial, the reduction of breast cancer incidence with tamoxifen treatment, was reached. Based on the overwhelming data that tamoxifen is an effective prophylactic breast cancer agent, the committee, based on ethical considerations, determined that the study be unblinded, thus allowing the placebo population of the trial consider tamoxifen treatment or enroll in a second prevention trial that compared tamoxifen with another SERM, raloxifene (Fisher 1999).

Overview analysis (Cuzick, Powles et al. 2003) of the four major chemoprevention trials showed 46% reduction in the rates of breast cancer incidence. Moreover, even though statistically significant increases of endometrial cancer rates were not observed in the tamoxifen treated group of all trials, a significant finding became apparent. Most of the endometrial cancer cases involved postmenopausal women. In addition to endometrial cancer, tamoxifen treated individuals had elevated risk of death caused by pulmonary embolisms and significant increase of thromboembolic events.

The potential public health impact of the NSABP P-1 trial is difficult to ascertain. Initial analysis (Fisher 1999) estimated that in a five year period approximately 500,000 invasive and 200,000 non invasive breast cancers could be prevented among the approximately 29 million women in the United States eligible for tamoxifen chemoprevention. Yet, subsequent analysis

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(Rockhill, Colditz et al. 2000) based on the findings of the NSABP P-1 trial and their application to the Nurses Health Study (Rockhill, Spiegelman et al. 2001), deemed these estimates high. Analysis (Freedman, Graubard et al. 2003) of nationally representative data from the year 2000 National Health Interview Survey (NHIS) tried to determine the benefits (reduction of invasive breast cancer and bone fractures) and risk of adverse events (increase in endometrial cancer and thromboembolic events) associated with tamoxifen treatment. The analysis concluded that 15.5% of women aged 35-79 in the Unites States would be eligible for tamoxifen chemoprevention, based on age and risk factors. However, the percentage of women eligible for chemoprevention varies with age. For example, 45% of white women over the age of 60 would be considered eligible for chemoprevention, but eligibility certainly does not translate into net chemoprevention benefit. Overall, from the 18.7% of white women eligible for chemoprevention, only 4.9% would receive a net benefit. Furthermore, even though the analysis indicates that the highest percentage of women eligible for chemoprevention is in the 60-79 years age group, the greatest percentage of white women who would benefit the most fall into the 40-49 and 50-59 year age groups.

The other issue to be considered with the availability of tamoxifen is efficacy based on compliance and cost. A recent study of tamoxifen compliance in the treatment setting found that over one third of women stopped taking tamoxifen, a proven therapy that aids survival, after 3-5 years (Barron, Connolly et al. 2007). Additionally, cost of chemoprevention is an issue for health services. It is estimated that only very high risk women (Gail score $\geq$3), those with a risk of few negative side effects, would benefit and only in an environment that provides cheap generic tamoxifen (Melnikow, Kuenneth et al. 2006).

The risk-benefit analysis of any prophylactic agent must be carefully examined with a focus on the overall patient population. As a result, new strategy (Jordan 1988) was initiated that would improve on the net benefits achieved with tamoxifen.

**Clinical evaluations of Raloxifene to prevent osteoporosis**

Overall, the story of the clinical development of raloxifene is the story of changing ideas about the relevance of models to predict population outcomes. The idea of a SERM is to address the prevention of three major diseases: osteoporosis, atherosclerosis and breast cancer. The goal was to replace HRT for the treatment of osteoporosis with a SERM to reduce breast cancer risk. Unfortunately, the idea that a decrease in circulating cholesterol observed with HRT and raloxifene would translate into lives saved from coronary heart disease (CHD) proved to be wrong (Mosca, Barrett-Connor et al. 2001; Rossouw, Anderson et al. 2002; Anderson, Limacher et al. 2004).

In pilot clinical trials, raloxifene was shown to lower serum cholesterol levels without increases in triglycerides or endometrial effects and decrease bone turnover, as determined by biochemical markers (Draper, Flowers et al. 1996). These findings further supported the hypothesis that an antiestrogen may be used for treatment of breast cancer and can have beneficial effects on number of other factors, including osteoporosis (Jordan 1988). The effects of various doses of raloxifene on BMD (regional and total), bone turnover markers, serum lipids and endometrial thickness were addressed in a two year clinical trial (Delmas, Bjarnason et al. 1997). The study population included 601 postmenopausal, 45-60 years old women with osteoporosis. The study groups received placebo, 30, 60 or 150 mg raloxifene daily supplemented with 400-600 mg elemental calcium. Serum lipids and bone turnover markers

were measured every 3 months while spine and hip BMD, as well as endometrial thickness, were measured every 6 months. Within 3 months of raloxifene treatments, as compared to placebo, decreased the levels of the bone turnover markers within the levels of healthy postmenopausal women. Furthermore, raloxifene treatments increased the lumbar spine, femoral neck, total hip and total body BMD. The population receiving the highest, 150 mg daily raloxifene dose had the greatest increase in all categories with exception of total hip BMD (60 mg dose had the greatest increase). Raloxifene treatments decreased the levels of LDLs and total cholesterol in dose dependent fashion without changes in the levels of HDLs and triglycerides. Raloxifene was relatively well tolerated and no differences in adverse events or proportion of women reporting hot flashes were observed between the placebo and raloxifene treatment groups. Most importantly no increases in endometrial thickness were observed in the raloxifene treated population. The positive clinical profiles obtained during the study indicated that raloxifene may be useful in the prevention of osteoporosis and cardiovascular disease, without negative effectss on the endometrium.

A subsequent clinical trial (Walsh, Kuller et al. 1998) examined the lowest effective dose of raloxifene on intermediate cardiovascular end points and compared the effects to those of hormone replacement therapy (HRT). The primary end points considered were the levels of HDL and LDL cholesterol, triglycerides and the clotting factor fibrinogen. The study population consisted of 390 healthy, postmenopausal women, 45-72 years old. The treatments included placebo, 60 or 120 mg daily raloxifene and HRT (conjugated equine estrogen 0.625mg daily and medroxyprogesterone acetate 2.5 mg daily). The duration of treatments was six months. Effects of treatment were apparent within the first three months and persisted during the duration of the study. LDL cholesterol levels, as compared to placebo, were decreased 12% with raloxifene

treatments and 14% with HRT. HDL cholesterol levels were not affected by raloxifene treatments but increased 10% with HRT. Triglyceride levels were also unaffected by raloxifene treatments but increased 20% with HRT. In contrast, raloxifene treatments decreased the levels of fibrinogen, while HRT did not affect the fibrinogen levels. The most common side effects reported were hot flashes, which were most common in the 120 mg raloxifene group. Overall, raloxifene had similar cardiovascular effects as HRT in healthy, postmenopausal women. Most importantly, the decrease of LDL cholesterol further indicated to investigators at the time, that raloxifene treatments may decrease the risk of coronary artery disease.

The Multiple Outcomes of Raloxifene Evaluation (MORE) trial (Ettinger, Black et al. 1999) was initiated to determine the effects of raloxifene therapy on the risk of vertebral and non-vertebral fractures. The study population consisted of 7,705 postmenopausal women, ages 31-80, with osteoporosis and the study population was subdivided into 2 subgroups. The first subgroup included women with femoral neck and lumbar spine BMD $t$ score <2.5. The second subgroup included women with low BMD and one or more moderate to severe vertebral fractures and women who had at least two moderate fractures regardless of BMD. The treatments included placebo and 60 or 120 mg raloxifene, supplemented by 500 mg calcium and 400-600 IU calciferol. The primary end points considered were incidental vertebral fractures and BMD. The secondary end point consisted of any non vertebral fractures. At the 36 month time point, overall and in each individual raloxifene treatment group, the raloxifene treated individuals had fewer new vertebral fractures. Similar rates of non vertebral fractures were observed for all study groups, with the exception of the statistically significant differences in ankle fractures between the pooled raloxifene groups and placebo. Femoral neck and spine BMD were increased and bone turnover markers were decreased in the raloxifene treated groups. No differences in

endometrial cancer rates were observed between the raloxifene and placebo treated individuals. However, significant increases of thromboembolic events (including deep vein thrombosis (DVT) and pulmonary embolisms) was observed among the raloxifene treated individuals. Therefore, raloxifene was considered to be a very well tolerated agent.

Forty eight month follow up (Delmas, Ensrud et al. 2002) indicated that raloxifene treatment significantly decreases the risk of vertebral fractures in both study subgroups, without significant differences between the two raloxifene doses. However, there were no indications that raloxifene treatment decreased the risk of non vertebral fractures. Similarly to the 36 month time point, continuous raloxifene treatment significantly improved the lumbar spine and femoral neck BMDs. It is important to note that 36 months was the primary end point of the MORE trial. An additional year of follow up was used primarily to determine the cumulative effects of raloxifene on vertebral fracture risks during a 4 year time period.

More importantly, the MORE trial provided an appropriate arena for testing the breast cancer chemoprevention concept (Lerner and Jordan 1990) that an "antiestrogenic" medicines, in this case raloxifene, may treat a disease caused by overall physiological changes during menopause, but also significantly reduce the development of breast cancer. Indeed, subsequent analysis (Cummings, Eckert et al. 1999) of the MORE trial participants indicated that during the 3 year MORE trial the raloxifene treated individuals had substantially lower rate of breast cancer. During 40 months median follow up period, the rate of all breast cancers was 4.3 cancers per 1000 women years in the placebo and 1.5 cancers per 1000 women years.

The rates of invasive breast cancers were 3.6 cancers per 1000 women years in the placebo and 0.9 invasive breast cancers per 1000 women years in the raloxifene pooled groups. It was determined that raloxifene decreased the risk of invasive ER positive breast cancer by 90%,

while the rate of invasive ER negative breast cancer remained constant, albeit with a high confidence interval. The positive effects of raloxifene on breast cancer were accompanied by negligible effects on the endometrium.

The Continuous Outcomes Relevant to Evista (CORE) (Ettinger, Black et al. 1999) trial was an extension of the MORE trial. It examined the effects of 4 years additional raloxifene treatment, on a subset of the population from the MORE trial. Therefore, the study population consisted of postmenopausal women with osteoporosis. The primary end point of the CORE trial was incidence of invasive breast cancer, while the secondary end point considered was the incidence of ER positive breast cancer. The treatments consisted of placebo and 60 mg daily raloxifene supplemented with 500 mg calcium and 400-600 IU Vitamin D. It is important to note that as the patient population had osteoporosis, the study population was allowed to take bone specific agents such as bisphosphonates, calcitonin or fluoride.

During the 4 years of the CORE trial the raloxifene treated individuals had 59% decreases in the incidence of invasive breast cancer. The incidence of invasive ER positive breast cancer was decreased by 66% in the raloxifene treated group. Most importantly, the incidence rate of invasive ER negative breast cancer was not changed by raloxifene treatment. Overall, raloxifene decreased the rate of all breast cancers by 50%. Analysis of the combined data from the MORE and CORE trials, indicated that after approximately 8 years (range 4.8-8.5 years), raloxifene treatment reduced the incidence of invasive breast cancer by 66%. The incidence of invasive ER positive breast cancers was decreased by 76% while the incidence of invasive ER negative cancer remained the same. Overall, regardless of ER status, raloxifene treatments decreased the incidence of breast cancer by 58%. The incidence of adverse effects, vaginal bleeding, endometrial hyperplasia and endometrial cancer were statistically insignificant between

the placebo and raloxifene groups in the CORE trial and during the combined duration of the MORE and CORE trials.

The MORE/CORE study demonstrated the effectiveness of raloxifene to reduce fractures while reducing the risk of breast cancer. However, it must be stated that there were periods between the two trials when women were not treated with raloxifene. As a result, it is possible that if compliance to raloxifene had been maintained, the risk of breast cancer could be reduced more effectively.

**Study of Tamoxifen and Raloxifene (STAR)**

The NSABP P-2 STAR trial was launched to compare the relative effects and safety of tamoxifen and raloxifene on the risk of developing invasive breast cancer in high risk populations of women (Vogel, Costantino et al. 2006). This study is an example of the distinct approach to chemoprevention in populations of postmenopausal women at high risk for breast cancer. Even though initially, and from a chronological aspect, the STAR trial appears to be a natural extension of the progress made in breast cancer chemoprevention with the MORE and CORE trials, in reality the STAR trial is an extension of the NSABP P-1 trial. The ethical considerations generated during the NSABP P-1 trial and the progress made during various adjuvant and chemopreventive tamoxifen trials laid the foundation for search for equivalent and/or superior breast cancer agents while minimizing undesired side effects.

The study population of the STAR trial was 19,747 healthy postmenopausal women with increased 5-year breast cancer risk. The treatments consisted of 20 mg daily tamoxifen and 60 mg daily raloxifene. The primary end point considered was invasive breast cancer. Secondary end points considered were diseases influenced by tamoxifen in previous breast cancer

prevention trials and included: endometrial cancer, *in situ* breast cancer, cardiovascular disease, stroke, pulmonary embolism, DVT, transient ischemic attack, osteoporotic fractures, cataracts, death and quality of life (Vogel, Costantino et al. 2006).

In regards to invasive breast cancer, both tamoxifen and raloxifene exhibited similar effects during the 6 years follow up (median follow up 3.9 years). There were no significant differences in the rates of invasive breast cancer between the tamoxifen and raloxifene groups.

The incidence of invasive breast cancer for the tamoxifen group was 4.3 cases per 1000 women years and 4.41 cases per 1000 women years for the raloxifene group. Overall, there were fewer cases of *in situ* breast cancer in the tamoxifen group than in the raloxifene group, and this finding was statistically insignificant but approaching significance (p=0.052). Tamoxifen and raloxifene also exhibited similar effects on uterine cancer. However, there was statistically insignificant trend of lower incidence of uterine cancer in the raloxifene group. Majority of uterine cancers occurred in women over 50 years of age. Even though the endometrial cancer rates were similar for both agents the raloxifene group had 38% lower incidence rate then the tamoxifen group. The incidence of uterine hyperplasia (with and without atypia) was decreased by 84% in the raloxifene group. Importantly, the number of hysterectomies during follow up in women not diagnosed with uterine cancer was significantly lower in the raloxifene group. No differences in the rates of other cancers were observed between the two groups. Lung cancers were more numerous in the raloxifene group but this finding was not statistically significant. Additionally, no differences in the incidence of ischemic heart disease, strokes and fractures were observed between the two groups. Significant differences between the two groups were observed in regards to thromboembolic events, cataracts and cataracts surgery. The raloxifene treated group had a 30% decrease in thromboembolic events (pulmonary embolisms and DVT)

and significantly less participants in the raloxifene group developed cataracts and had cataracts surgery.

The STAR trial indicates that raloxifene and tamoxifen are agents with similar breast cancer chemopreventive efficacy. Although the differences between the two treatment groups were not statistically significant in regards to invasive breast cancer, there were fewer instances of non invasive breast cancer in the tamoxifen group, indicating that tamoxifen may be a more efficient agent in prevention of non invasive breast carcinoma. However, even though both drugs exhibited similar chemopreventive efficacy, raloxifene exhibited a superior safety profile.


**Raloxifene Use for the Heart (RUTH)**

Tamoxifen (Love, Newcomb et al. 1990) and raloxifene (Delmas, Bjarnason et al. 1997; Ettinger, Black et al. 1999; Delmas, Ensrud et al. 2002) therapy has been associated with positive changes in various cardiovascular markers. These observations raised the possibility that a potential side effect of SERM therapy may be an improved cardiovascular system. Combination of these findings with the observations that HRT may not significantly decrease the incidence of CHD in post menopausal women (Hulley, Grady et al. 1998; Rossouw, Anderson et al. 2002; Anderson, Limacher et al. 2004) led to the RUTH trial (Barrett-Connor, Mosca et al. 2006). The purpose of the RUTH trial was to determine the effects of raloxifene on cardiovascular events as compared to placebo. The trial included 10,101 postmenopausal women with established risk of CHD. The treatments included placebo and 60 mg daily raloxifene. When all combined coronary end points were considered there were no differences between the two groups. Additionally, no difference in the overall stroke incidence was observed. Nevertheless, the incidence of fatal stroke was 49% higher in the raloxifene group. Significant differences between the groups were

observed in regards to venous thromboembolic events (44% higher in the raloxifene group), breast cancer (33% lower incidence of all breast cancers) and clinical vertebral fractures (35% lower incidence in the raloxifene group). Most importantly, the incidence of endometrial and other cancers did not differ between the groups. No differences in the numbers of adverse events were observed between the two groups, but a significantly larger number of women in the raloxifene group discontinued therapy due to adverse events.

Overall, extended raloxifene therapy (median 5.6 years) did not provide any significant cardiovascular benefits but while significantly decreasing the rates of breast cancer and clinical vertebral fractures, it significantly increased the number of venous thromboembolic events. Concurrently, large clinical trials (Hulley, Grady et al. 1998; Rossouw, Anderson et al. 2002; Anderson, Limacher et al. 2004) of hormonally treated postmenopausal women have failed to demonstrate any benefits of estrogen therapy on cardiovascular health. These findings, though disappointing from a cardiovascular standpoint, further reinforce the need to further develop the SERM concept. Current trials support the idea that SERMs decrease the risk of breast cancer, and raloxifene (but not tamoxifen) does not increase the risk of endometrial cancer in postmenopausal women. Additionally, SERMs reduce the incidence of vertebral fractures in postmenopausal women, and raloxifene has a more favorable safety profile than tamoxifen in postmenopausal women. These clinical observations have now established a new drug group into medicine and it is appropriate to conclude with a discussion of their potential use in clinical practice.


**Conclusions**

Three issues are important to optimize the process of breast cancer chemoprevention: a) identification of the target population; b) selection of an appropriate agent; and c) the burden of the cost of chemopreventive therapies on public health systems.

The Gail model (Gail, Brinton et al. 1989) has been successfully used for identification of patient populations in the NSABP P-1 and STAR P-2 trials. However, in the Nurses Health Study (Rockhill, Spiegelman et al. 2001), a study involving over 80,000 women, 44% of the observed breast carcinomas occurred in the high risk group (Gail risk of ≥1.67) and 54% of the breast cancers occurred in population of women deemed not at risk for breast cancer as predicted by the Gail model. Therefore, it is important to develop models that could identify the desired target populations and distinguish various degrees of risks within the patient populations.

The link between ovarian hormones and breast cancer was noted over a century ago (Beatson 1896) and the idea that creating a no estrogen state may prevent breast cancer was suggested approximately 70 years ago (Lacassagne 1936). Aromatase inhibitors that are currently used to treat breast cancer use this concept and current clinical trials have shown their superiority over tamoxifen in inhibiting contralateral breast cancer in postmenopausal women (Coombes, Hall et al. 2004; Goss, Ingle et al. 2005; Howell, Cuzick et al. 2005; Thurlimann, Keshaviah et al. 2005). However, what would be the cost, both to women and health care systems, if aromatase inhibitors are the agent of choice?

If one assumes a population similar to those described in the STAR trial (Vogel, Costantino et al. 2006) and identifies a high risk population of postmenopausal women based on the Gail model, then the incidence of breast cancer will be 8 per 1000 women annually. Chemopreventive application of aromatase inhibitors in the patient population may prevent three out of four breast cancers. Therefore, in order to prevent 6 breast cancers, an additional 992

women will need to be treated without other benefits and with potential for harmful side effects. Based on the National Health and Nutrition Examination Survey, up to 18% of women over the age of 50, in the United States suffer from osteoporosis and up to 50% suffer from osteopenia (Looker, Orwoll et al. 1997). Aromatase inhibitors will increase a woman's risk for osteoporosis and thus other, alternative preventive strategies need to be considered.

The concept of chemoprevention with SERMs has been developed, tested in the laboratory and refined over the past 20 years. The evidence based laboratory concept (Jordan 1988) has been successfully tested in the clinic and can now be used to extrapolate the results of the raloxifene clinical studies to estimate public health benefits. The MORE trial (Cummings, Eckert et al. 1999) established the initial proof of principle that a SERM could be successfully used to prevent osteoporotic fractures in a postmenopusal population while at a same time decreasing the breast cancer rate. The CORE trial (Martino, Cauley et al. 2004) further documented a significant decrease in the breast cancer rates during long term (up to 8 years) raloxifene treatments. However, it is interesting to point out that in contrast to the MORE and CORE trials that recorded 65-75% decrease in the breast cancer rates; there was only an estimated 50% decrease in the rates of breast cancer during the STAR trial. One reason for such a discrepancy may be the target population of the respective trials. Raloxifene may perform exceptionally well in low estrogen states such as those observed in osteoporotic women. This was the patient population in the MORE and the CORE trials. In contrast, the patient population of the STAR trial consisted of healthy postmenopausal women with possibly higher levels of circulating estrogen. Additional factors for such discrepancies may be low patient compliance combined with the raloxifene's poor bioavailability (Gottardis and Jordan 1987; Snyder, Sparano et al. 2000; Jordan 2006). Raloxifene's poor bioavailability illustrates the need for long lasting

SERMs and indeed, new long lasting alternatives such as arzoxifene (Sato, Turner et al. 1998; Suh, Glasebrook et al. 2001), may become available in the near future. Arzoxifene is superior to raloxifene in prevention of rat mammary carcinogenesis (Suh, Glasebrook et al. 2001) and clinical trials for the treatment and prevention of osteoporosis are nearing completion. Thus a SERM that reduces breast and endometrial cancers while increasing bone density will be a suitable intervention to prevent breast cancer in postmenopausal, high and low risk, women. The fact that SERMs are cheaper then aromatse inhibitor is also an advantage. But what of tamoxifen, the veteran SERM?

Tamoxifen is available in the US as an effective chemopreventive agent in a high risk postmenopusal population; however there is significant increase in endometrial cancer. Evaluation of mortality outcomes (Melnikow, Kuenneth et al. 2006) have projected that the use of tamoxifen in populations with Gail risk ≥3 will have maximum benefit, but only in countries with affordable tamoxifen. Use of tamoxifen, particularly in managed health care systems, must be accompanied with comprehensive patient follow up due to tamoxifen's significant side effects. Naturally, tamoxifen is a viable option in hysterectomized women. Nevertheless, tamoxifen is the only agent available to reduce the risk of breast cancer in premenopausal women. There are no elevations in endometrial cancer or blood clots in premenopausal women making tamoxifen a reasonable health choice. Compliance is also a major consideration for healthy women taking a medicine that decreases quality of life. In the case of tamoxifen, a large proportion of women report increase of hot flashes and menopausal symptoms. In recent years physicians have prescribed selective serotonin reuptake inhibitors (SSRIs) that significantly reduce hot flashes. However, the finding that tamoxifen must be converted to an active metabolite, endoxifen, for optimal activity and that some of the SSRIs block the CYP2D6

enzyme responsible for that conversion, is of concern (Jordan 2007) (Figure 1). Also, there are individuals with non functional alleles (CYP2D6 *4/*4) that have no enzymatic activity. It is therefore reasonable that if a woman is to complete 5 years of treatment for chemoprevention, she should determine whether she has an aberrant CYP2D6 enzyme and is not taking SSRI known to impair tamoxifen metabolism (Stearns, Johnson et al. 2003; Goetz, Rae et al. 2005; Goetz, Knox et al. 2007).

In closing, it is now possible to recommend a practical strategy to patients to reduce the risks of breast cancer. Twenty years ago this was not possible. The SERM concept (Jordan 1988) has provided clues for further research development strategies for other members of the steroid receptors super family (Smith and O'Malley 2004). Anabolic androgens that do not stimulate the prostate would be valuable medicines. Alternatively, glucocorticoids that can control inflammation without causing bone loss would be invaluable. A dedicated program of drug discovery and development is now possible to create targeted therapies previously thought to be impossible.

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**Table 1 A comparison of patient characteristics in the tamoxifen prevention trials**

| Population characteristics | Royal Marsden | Italian | IBIS-I | NSABP P-1 |
|---|---|---|---|---|
| Study size | 2471 | 5408 | 7169 | 13388 |
| Participants >50 years old | 62% | 36% | 49% | 40% |
| Median follow up | 70 months | 48 months | 50 months | 54.6 months |
| 1° relative with breast cancer | 55% | 18% | 48.1% | 55% |
| > 1° relatives with breast cancer | 17% | 2.5% | 61.7% | 13% |
| Use of HRT | 41% | 8% | 41% | 0% |
| **Breast cancer incidence per 1000 individuals** | | | | |
| Placebo | 5.5% | 2.3 | 6.74 | 6.7 |
| Tamoxifen | 4.7% | 2.1 | 4.58 | 3.4 |

**Figure 1.** The metabolism of tamoxifen to active hydroxylated metabolites thought to play a significant role in the antiestrogenic and anticancer actions of tamoxifen. The P450 enzyme CYP2D6 is important to produce the metabolite endoxifen but the selective serotonin reuptake inhibitors (SSRIs) paroxetine and fluoxetine bind strongly to CYP2D6 and block endoxifen production. The SSRIs are used to reduce hot flashes in women taking tamoxifen. In contrast, the SSRI venlafaxine has a low affinity for CYP2D6 and is the preferred treatment for hot flashes.

Word Count: 5487

# A Century of Deciphering the Control Mechanisms of Estrogen Action in Breast Cancer: the origins of targeted therapy and chemoprevention

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**Short title:** Hormone dependent breast cancer

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**Key Words:   breast cancer, estrogen receptor, tamoxifen, antiestrogen, selective estrogen receptor modulators, antiandrogens, prostate cancer**

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**Abstract**

The origins of the story to decipher the mechanisms that control the growth of sex hormone dependent cancers started more than a hundred years ago. Clinical observations of the apparently random responsiveness of breast cancer to endocrine ablation (hormonal withdrawal) provoked scientific enquiries in the laboratory that resulted in the development of effective strategies for targeting therapy to the estrogen receptor (ER) (or androgen receptor in the case of prostate cancer), the development of antihormonal treatments that dramatically enhanced patient survival and the first successful testing of agents to reduce the risk of developing any cancer. Most importantly, elucidating the receptor mediated mechanisms of sex steroid dependent growth and the clinical success of antihormones has had broad implication in medicinal chemistry with the synthesis of new selective hormone receptor modulators for numerous clinical application. Indeed, the successful translational research on the ER was the catalyst for the current strategy for developing targeted therapies to the tumor and the start of "individualized medicine". Over the past fifty years, ideas about the value of antihormones translated effectively from the laboratory to improve clinical care, improve national survival rates and significantly reduced the burden of cancer.

**Beginnings at the dawn of the 20th Century**

Schinzinger (1) is credited with suggesting that oophorectomy could be used to treat breast cancer, however, this suggestion did not appear to have been adopted. In contrast, the report by Beaston (2) that oophorectomy could initiate a regression of metastatic breast cancer in two premenopausal women was a landmark achievement. Although it is often stated that Beaston's work was empirical clinical research, the rationale to conduct an oophorectomy was, in fact, an example of early translational research. Beaston was aware of the essential role of removing the ovary in maximizing milk production in cows. He reasoned there was potentially some factor that traveled in the blood supply to the breast as there was no known connection through the nerves. Interestingly enough, he also conducted laboratory experiments in rabbits before his clinical experiment, so the work was bench to bedside (2). By 1900, Stanley Boyd (3) had assembled the results of all the available clinical cases of oophorectomy to treat breast cancer in Great Britain in perhaps the first "clinical trial". Boyd concluded that only a third of metastatic breast tumors responded to oophorectomy. This clinical result and overall response rate has remained the same to this day.

Unfortunately, responses were of limited duration and enthusiasm waned that this approach was the answer to cancer treatment. Also, the approach of endocrine ablation was only relevant to breast cancer (and subsequently prostate cancer (4)) thus, the approach was only effective in a small subset of cases of all cancer types. At the dawn of the 20th Century, there was no understanding of the endocrine system or hormones. Nevertheless, laboratory studies started to decipher the biological control mechanisms responsible for the clinical observations.

**Links between sex steroids and cancer**

The fashion in breast cancer research in the early years of the 20th century was to use inbred strains of mice to study the growth and incidence of spontaneous mammary cancer. Lathrop and Loeb (5) found that before three months of age was the optimal time for oophorectomy to **prevent** the development of mammary cancer, but obviously this knowledge could not be translated to the clinical setting; who would one treat? The mechanism was also unknown until Allen and Doisy (6), using an ovariectomized mouse vaginal cornification assay demonstrated that a principle, that they called estrogen (identified as estrone the principal steroid), was present in ovarian follicular fluid. Their major advance set the scene for the subsequent breakthroughs in molecular endocrinology and therapeutics in the latter half of the 20th century (Figure 1).

The idea that breast cancer might be a preventable disease was extended by Professor Antoine Lacassagne who first demonstrated that estrogen could induce mammary tumors in mice (7, 8). Lacassagne (9) hypothesized *"If one accepts the consideration of adenocarcinoma of the breast as the consequence of a special hereditary sensibility to the proliferative action of oestrone, one is led to imagine a therapeutic preventive for subjects predisposed by their heredity to this cancer. It would consist – perhaps in the very near future when the knowledge and use of hormones will be better understood – in the suitable use of a hormone, antagonistic or excretory, to prevent the stagnation of oestrone in the ducts of the breasts."* However, when Lacassagne stated his vision at the annual meeting of the American Association for Cancer Research (Boston) in 1936, there were no lead compounds that antagonized estrogen action but the Allen Doisy mouse assay could be used to study structure activity relationships to find

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synthetic estrogens.  Within a decade, a landmark discovery was to occur in "chemical therapy" that was to expand the treatment of metastatic breast cancer to include postmenopausal women who are in fact the majority who develop metastatic disease.

During the 1930's there were significant advances in the knowledge of the precise structural requirements for estrogen action in its target tissue, the vagina.  Synthetic compounds based on stilbene (10, 11) and triphenylethylene (12)  were screened using the Allen Doisy ovariectomized mouse vaginal cornification assay to define compounds with optimal structures and duration of estrogen action. Sir Alexander Haddow found that carcinogenic polycyclic hydrocarbons would cause tumor regression in animals. However, these could not be used to treat humans.  The nonsteroidal triphenylethylene-based estrogens had similar structures to polycyclic hydrocarbons and also caused tumor regression in animals. With this clue, Sir Alexander Haddow used the first chemical therapy to treat patients.  His resulted published in 1944 (13) demonstrated that high dose estrogen therapy was effective in causing tumor regressions in postmenopausal patients with breast cancer and men with prostate cancer. There was, however, no understanding of a mechanism.   Indeed he stated in 1970: *In spite of the extremely limited practicability of such measure [high dose estrogen], the extraordinary extent of tumor regression observed in perhaps 1% of post-menopausal cases has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us*" (14).  These experimental data were also an apparent paradox as endocrine ablation to **remove** estrogens and their precursors was the dogma of the time (15).

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In the past 50 years, the progress in deciphering the control mechanisms of estrogen action in breast cancer (and androgen action in prostate cancer), has accelerated with advances in technology and an understanding of cell biology. But progress in research does not travel in straight lines but chance observations can create a major breakthrough. This has happened repeatedly in the story of the treatment and prevention of breast cancer.

**Conceptual progress through scientific serendipity**

It is perhaps relevant to illustrate a few astute observations by scientists that accelerated progress immensely in deciphering the complexities of hormone action and the control of breast cancer growth.

Sir Charles Dodds is credited with the synthesis of the potent synthetic estrogen diethylstilbestrol (11) (Figure 2) that was subsequently used for the treatment of both prostate cancer and breast cancer and regrettably was also applied to prevent recurrent abortions (16) which caused a rise in clear cell carcinoma of the vagina in the children (17). During the race to describe the minimal molecular structure that would trigger vaginal cornification in the ovariectomized mouse vagina, controversy erupted in the 1930's over the reproductivity of results concerning the compound anethole. The authors were minimalistic in reporting the synthetic methodology so replication proved impossible to create the correct biology. Rather the product was correct, but the method used by the *original* authors were not reported accurately and actually caused dimerization of anethole to an impurity dianethole an estrogen. This active impurity was structurally similar with the parallel research endeavors that concluded with the synthesis

of the potent estrogen diethylstilbestrol. Thus, the purity of chemicals for testing was critical for successful science.

A similar story was also immensely important in allowing scientists to understand the direct actions of estrogen on the breast cancer cell *in vitro*. The MCF-7 ER positive breast cancer cell line (18) has been the work horse for the study of estrogen-stimulated growth. However, early examination of MCF-7 cells in the 1970's could not uniformly demonstrate estrogen stimulated growth. Antiestrogens inhibited the apparently constitutive growth of MCF-7 cells but estradiol did reverse the inhibitory actions of antiestrogens on growth (19). The mystery deepened when studies *in vitro* could not demonstrate estrogen-stimulated growth but MCF-7 cells inoculated into athymic mice would grow into tumors only with estrogen treatment. There was clearly a second factor required for estrogen-stimulated tumor growth *in vivo*! (20).

The astute observations of John and Benita Katzenellenbogen solved the mystery of why estrogen did not stimulate MCF-7 breast cancer cell growth *in vitro*. It appears that all cells had been grown for more than a decade in standard media containing large concentrations of a pH indicator called phenol red. The Katzenellenbogens realized that the structure of phenol red was similar to non-steroidal estrogens and removal of the indicator from cell culture media caused cell growth rate to fall and only then would exogenous estrogen cause growth (21). In other words, the cells were already growing maximally in phenol red containing media. Subsequent studies revealed that the culprit was, in fact, a partially dimerized chemical contaminant of phenol red. This critical technical advance permitted all of the subsequent understanding of the molecular biology of direct estrogen action.

Leonard Lerner was a young research endocrinologist employed by Merrell Dowe to study nonsteroidal estrogen pharmacology. He noticed that the structure of one of the compounds being tested for the control of coronary artery disease was a triphenylethanol similar to the estrogenic triphenylethylenes and he asked to test this chemical as an estrogen. To his surprise the compound, subsequently renamed MER25 or ethamoxy-triphetol was antiestrogenic in all species tested and had no estrogen-like actions in any animal tests (22). Lerner had discovered the first nonsteroidal antiestrogen (22). Although the compound was too toxic and not potent enough for clinical use, Lerner went on to be involved in the discovery of the first triphenylethylene antiestrogen called chloramiphene (MRL41) later to be known as clomiphene (23). Originally, the nonsteroidal antiestrogens were predicted, based on animal studies, to be potent postcoital contraceptives, which in the early 1960's had a huge potential market as "morning after pills". However, clomiphene did exactly the opposite; it induced ovulation in women (23). Enthusiasm waned and there was general disinterest in this area of research until ICI 46,464 another non-steroidal antiestrogen discovered in the fertility program of ICI Pharmaceutical Ltd (now AstraZeneca) (24) was reinvented as the first targeted therapy for breast cancer and the first chemopreventive for any cancer (25)

**A target for treatment and prevention**

The early theory for estrogen action in its target tissues e.g. uterus, vagina, etc., was that there was chemical transformation between estrone and the less abundant 17β estradiol (Figure 2) to control the redox potential of the tissue environment. In the late 1950's, Jensen (Figure 3) and Jacobsen (26) chose another approach at the Ben May

Laboratories of the University of Chicago. They synthesized (6, 7) [$^3$H] estradiol (Figure 2) with very high specific activity and following injection into the immature female rats, the unchanged steroid bound to and was retained by the estrogen target tissues, the uterus, vagina and pituitary gland. In contrast, [$^3$H] estradiol bound to but was not retained by non target tissues e.g. muscle, lung, heart. There was clearly a receptor mechanism at play that could be blocked (27) by the co-administration of the first nonsteroidal antiestrogen MER-25 (22).

The mystery of why only about a third of advanced breast cancers responded to either endocrine ablation (3) or high dose estrogen therapy (15) was solved by the application of basic endocrinology to the practical issue of excluding women with metastatic breast cancer who **would not** significantly benefit from unnecessary endocrine ablative surgery (oophorectomy, adrenalectomy or hypophysectomy). The estrogen receptor (ER) was found to be an extractable protein from the rat uterus that would bind [$^3$H] estradiol in the extraction cytosol (28, 29). During the late 1960's, numerous methods were described to identify and quantitative ER levels in tumor biopsies (30) and these data were subsequently correlated with clinical outcomes in metastatic breast cancer (31). Breast tumors without the ER were unlikely to respond to endocrine ablation and therefore should not be treated with this modality. The ER assay was introduced as the standard of care in the mid 1970's to predict endocrine responsiveness to endocrine ablation. It should be stressed that tamoxifen was not available in medical practice until the FDA approved this "hormone therapy" in December 1977 for the treatment of metastatic breast cancer in postmenopausal women (23). Indeed, research with the value of the ER assay to predict responsiveness to antiestrogens was

unconvincing (23) and the value of adding another "hormone therapy" to the treatment armamentarium was uncertain. The 1970's was a time when all hopes in medical oncology were focused on discovering the correct combination of high dose cytotoxic therapies to cure breast cancer much in the same way as both childhood leukemias and Hodgkin's Disease had been cured. This was not to be but translational research took another route using the ER as a drug target and not as a predictive test for endocrine ablation (32).

**An unlikely therapeutic solution**

Professor Paul Ehrlich (1854-1915) established a model for the development of chemical therapies (chemotherapy) to treat infectious disease. A range of chemical therapies would be synthesized to study structure function relationships in appropriate laboratory models that replicated human disease (33). A clinical study would then be performed on the most promising candidate. Ehrlich's pioneering work to develop Salvarsan for the successful treatment of syphilis is a landmark achievement (33). He was, however, unsuccessful in applying the same principles to cancer chemotherapy. Indeed, even as recently as 1970, Sir Alexander Haddow (14) stated that there was unlikely to be a "chemotherapia specifica" in the sense that Paul Ehrlich envisioned because cancer was so similar to the tissue of origin. There was also no target or effective tests or models to predict efficacy in cancer treatment prior to administration to the patient. The key to the successful development of tamoxifen, a failed contraceptive, (23) was the application of Ehrlich's principles of developing an effective treatment

strategy by employing disease specific laboratory models and the utilization of the tumor ER as a target for drug action (25).

Available laboratory models for the study of the antitumor actions of antiestrogenic drugs were strains of mice with a high incidence of spontaneous mammary tumors (5) or the carcinogen-induced rat mammary carcinoma (34). The mouse models had fallen out of fashion with the discovery of the "Bittner milk factor" a virus that transmits mammary carcinogenesis to subsequent generations through the mother's milk (35). The research community also began to realize that breast cancer was not a viral disease. Nevertheless, the knowledge of mouse mammary carcinogenesis proved to be pivotal for developing precise and targeted promoters to initiate mammary cancer with oncogenes using transgenic mice (36). Another problem with tumor testing of tamoxifen in mice was the unusual observation that tamoxifen, or ICI 46,474 as it was then known, was an estrogen in the mouse (24, 37). This pharmacological peculiarity was later to become important with the recognition of selective ER modulation (38). Most importantly, work did not advance quickly in the 1960's and early 1970's, as there was no enthusiasm about introducing a new "hormonal therapy" into clinical practice (25). All early compounds had failed to advance past early clinical studies and only tamoxifen was marketed (23) for the induction of ovulation or the general treatment of late stage breast cancer in postmenopausal women (39-41).

In the late 1960's, the 7, 12 dimethybenz[a]anthracene-induced (DMBA) rat mammary carcinoma model (34) was extremely fashionable for research on the endocrinology of rat mammary carcinogenesis (42, 43). However, the parallels with breast cancer are few as the tumors do not metastasize and are regulated primarily by

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prolactin secreted by the pituitary gland in direct response to estrogen action (44).  Be that as it may, there was no alternative so the DMBA rat mammary carcinoma model was adapted to determine the appropriate strategy for the use of antihormonal therapy as an adjuvant.  At that time in the mid-1970's, the early adjuvant trials with tamoxifen did not target patients with ER positive breast cancer and used only short term (1 year) tamoxifen treatment to avoid premature drug resistance.  This duration of tamoxifen was selected as the antiestrogen only controlled the growth of metastatic breast cancer for about a year (40).  The value of short and long term (1 or 6 months treatment equivalent to 1 or 6 years of adjuvant treatment in patients) antihormone administration was determined starting treatment one month after DMBA administration to 60 day old Sprague Dawley rats.  Long term therapy was remarkably effective at controlling the appearance of mammary tumors and was far superior to short term treatment (45, 46).  The concepts of targeting the ER and using long term adjuvant therapy effectively translated through clinical trials to improve national survival rates for breast cancer. (47, 48).

**Targeting treatment for breast cancer**

The early clinical work of Santen (49) established the practical feasibility of employing aminogluthemide, an agent that blocks both adrenal steroidogenesis and the CYP19 aromatase enzyme to stop conversion of testosterone and androstenedione to estradiol and estrone respectively.  Unfortunately, aminogluthemide must be given with a natural glucocorticoid therefore long term therapy is not a practical possibility.  Brodie and coworkers (50, 51) advanced knowledge of the specific targeting of the CYP19 aromatase enzyme with the identification and subsequent development of 4 hydroxyandrostenedione (52) as the first practical suicide inhibitor of the aromatase

enzyme (Figure 4).   Incidentally, the pivotal work with both tamoxifen and 4-hydroxyandrostenedione (Figure 2 and 4) was initiated at the Worcester Foundation for Experimental Biology in Massachusetts in the early 1970's (53).  Brodie's contribution eventually became the catalyst to create a whole range of agents (e.g. anastrozole (Figure 3)) targeted to the aromatase enzyme for the treatment of breast cancer in postmenopausal women (54).   The clinical application of aromatase inhibitors has reduced the side effects noted with tamoxifen in postmenopausal women such as blood clots and endometrial cancer and there has been a small but significant improvement in disease control for the postmenopausal patient when results are compared with tamoxifen (55, 56).

However, recent research into the pharmacogenetics of tamoxifen has suggested that CYP2D6 enzyme product is important for metabolism to the active antiestrogen endoxifene (4-hydroxy-N-desmethyltamoxifen) (57) and the use of certain selective serotonin reuptake inhibitors (SSRIs) to reduce hot flashes appears to be contraindicated because of drug interaction at the CYP2D6 enzyme (58, 59).  Current research is also exploring the hypothesis that a mutated and ineffective CYP2D6 gene product undermines the therapeutic activity of tamoxifen (58, 59).  It may be that patients could eventually be selected for optimal effective tamoxifen treatment in cases of ER positive breast cancer.  This would be worth while for the chemoprevention of breast cancer. Clearly, the identification of patients for optimal long term use of tamoxifen should exclude those high risk women with a mutant CYP2D6 gene who choose to use chemoprevention, as tamoxifen treatment may possibly be suboptimal.

**Chemoprevention of breast cancer**

In the middle of the 1970's, Sporn (60) advanced the concept of the chemoprevention of cancer and strongly advocated this approach as the optimal and clearly most rational way to reduce the burden of cancer. Practical chemoprevention articulated by Lacassagne (9) has its foundations with the finding that tamoxifen prevents DMBA-induced rat mammary carcinogenesis (61, 62). These laboratory findings (46, 61, 62) and the subsequent clinical finding that adjuvant tamoxifen treatment reduces the incidence of contralateral breast cancer (63) prompted Powles (64, 65) to initiate the first exploratory trial to test the worth of tamoxifen to reduce the incidence of breast cancer in high risk women. Although numbers were small, the Powles study did ultimately demonstrate the ability of tamoxifen to reduce breast cancer incidence many years after the treatment had stopped (66). In contrast, the large study by Fisher (67, 68) definitively demonstrated the efficacy of tamoxifen to reduce the incidence of ER positive breast cancer initially and continues to do so after therapy stops in both pre and postmenopausal women at high risk. Tamoxifen became the first medicine approved by the Food and Drug Administration for risk reduction of any cancer. However, concerns, based on laboratory findings (69), about the potential of tamoxifen to increase the risk of endometrial cancer in postmenopausal women and the carcinogenic potential of tamoxifen as a hepatocarcinogen (70) demanded that there had to be a better way to reduce the risk of breast cancer as a public health initiative.

The recognition of SERM action by nonsteroidal antiestrogens that stimulate some estrogen target tissues but block estrogen stimulated tumor growth in others (71) introduced a new dimension into therapeutics and advanced chemoprevention. Raloxifene has its origins as a nonsteroidal antiestrogen for the treatment of breast cancer (72, 73) as

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LY156758 or keoxifene. The drug failed in that indication (74) and further development was abandoned (75). The discovery that both tamoxifen and keoxifene would maintain bone density in ovariectomized rats (76), block rat mammary carcinogenesis (77) but that keoxifene was less estrogen-like than tamoxifen in the rodent uterus (72) and was less effective in stimulating the growth of endometrial cancer (78) suggested a new therapeutic strategy (79). Simply stated (80): *"We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high-risk group to prevent breast cancer."*

Several years later, keoxifene was renamed raloxifene (Figure 2) and was shown to maintain bone density in osteoporotic or osteopenic women (81), and simultaneously reduce the incidence of invasive breast cancer without causing an increase in the incidence of endometrial cancer (82). Raloxifene went on to be tested against tamoxifen in the Study of Tamoxifen and Raloxifene (STAR) trial (83) and was FDA approved both for the treatment and prevention of osteoporosis in postmenopausal women and for the reduction of invasive breast cancer incidence in postmenopausal women at elevated risk. The clinical advances with SERMS to modulate estrogen target tissues has provided

exceptional opportunities to treat and prevent multiple diseases. However, for the future

it is the study of the molecular events of estrogen action that hold the promise of further

breakthroughs in patient care.

**Molecular mechanisms of estrogen and SERM action**

It is not possible to provide a review of the explosion of interest in receptor

mediated molecular mechanisms of action of estrogen so the reader is referred to

significant reviews to appreciate the evolution of the topic (84, 85). What will be

presented is an evolving guide to current thinking. There are two ERs referred to as α

and β (Figure 5 and 6). The receptor ERα is the traditional ER (26, 28) but it should be

stressed that the development of monoclonal antibodies to ER (86) was the essential step

for ERα cloning (87, 88) that provided the clues to discover ERβ (89). The receptor

proteins encode on different chromosomes and have homology as members of the steroid

receptor superfamily but there are distinct patterns of distribution and distinct and subtle

differences in structure and ligand binding affinity. An additional dimension that may be

significant for tissue modulation is the ratio of ERα and ERβ at a target site. A high ERα:

ERβ ratio correlates well with very high levels of cellular proliferation whereas the

predominance of functional ERβ over ERα correlates with low levels of proliferation (90,

91). The ratio of ERs in normal and neoplasic breast tissue may be an important factor

for the long-term success of chemoprevention with SERMs. There is, as a result, much

interest in synthesizing ER subtype specific ligands

There are functional differences between ERα and ERβ that can be traced to the

differences in the Activating Function 1 (AF-1) domain located in the amino terminus of

the ER (Figure 6). The amino acid homology of AF-1 is poorly conserved (only 20%).

In contrast, AF-2 region located at the C terminus of the ligand binding domain, differs only by one amino acid: D545 in ERα and N496 in ERβ . Since the AF-1 and AF-2 regions are critical for the interaction with other co-regulatory proteins and gene transcription, the structural differences between AF-1 provides a clue about the potential functional differences between ER α and β. Studies using chimeras of ER α and β by switching the AF-1 regions demonstrate that this region contributes to the cell and promoter specific differences in transcriptional activity. In general, SERMs can partially activate engineered genes regulated by an estrogen response element through ERα but not ERβ (92, 93) . In contrast, 4-hydroxytamoxifen and raloxifene can stimulate activating protein-1 (AP-1) regulated reporter genes with both ERα and ERβ in a cell dependent fashion.

The simple model for estrogen action, with either ERα or ERβ controlling estrogen regulated events, has now evolved into a fascinating mix of protein partners that have the potential to modulate gene transcription (Figure 5). It is more than a decade since the first steroid receptor coactivator (SRC-1) was first described(94). Now dozens of coactivator molecules are known and also corepressor molecules exist to prevent the gene transcription by unliganded receptors(95).

It is reasonable to ask how does the ligand program the receptor complex to interact with other proteins? X-ray crystallography of the ligand binding domains of the ER liganded with either estrogens or antiestrogens demonstrates the potential of ligands to promote coactivator binding or prevent coactivator binding based on the shape of the estrogen or antiestrogen receptor complex(96, 97). Evidence has accumulated that the broad spectrum of ligands that bind to the ER can create a broad range of ER complexes

that are either fully estrogenic or antioestrogenic at a particular target site(98). Thus a mechanistic model of estrogen action and antiestrogen action (Figure 5) has emerged based on the shape of the ligand that programs the complex to adopt a particular shape that ultimately interacts with coactivators or corepressors in target cells to determine the estrogenic or antiestrogenic response respectively. But how does transcription become initiated?

Not surprisingly, the coactivator model of steroid hormone action has now become enhanced into multiple layers of complexity thereby amplifying the molecular mechanisms of modulation (99). Associated molecules in the complex at the estrogen regulated promoter site causes the complex to be built and then destroyed in a dynamic cycle of DNA remodeling that initiates transcription. The cyclical stimulation of activated receptor complexes appear to be necessary to orchestrate a consistent estrogenic response at a target gene.

**Drug resistance to SERMs**

The acceptance of the concept of long term antihormonal therapy to target, treat, and prevent breast cancer(25) raised the specter of drug resistance to SERMs. However, the early models of SERM resistance did not reflect the majority of clinical experience. The natural laboratory models of antihormone resistance caused stimulation of tumor growth during a year of therapy (100) and therefore reflected drug resistance in patients with metastatic breast cancer who are only treated successfully for a year. However, the earlier laboratory models of drug resistance did not replicate clinical experience with adjuvant therapy for five years. Remarkably, drug resistance evolves (Figure 7) and the

survival signaling pathway in tamoxifen resistant tumors becomes reorganized so that instead of estrogen being a survival signal, physiologic estrogen now inhibits tumor growth (101). This discovery provides an invaluable insight into the evolution of drug resistance to SERMS and prompted the re-classification of the process through Phase I (SERM/estrogen stimulated) to Phase II (SERMs stimulated/estrogen) inhibited growth (102).

This model would also explain the earlier observations (13) why high dose estrogen therapy was only effective as a treatment for breast cancer in women many years after the menopause. Natural estrogen deprivation had occurred. The process is accelerated and enhanced, however, in patients treated long term with SERMs or aromatase inhibitors so that only low doses of estrogen are necessary to cause experimental tumors to regress. The new knowledge of the apoptotic action of estrogen (or androgen – see next section) could potentially lead to the discovery of a precise apoptotic trigger initiated naturally by steroid hormone receptors (102). Discovery of this apoptotic trigger might result in applications to target critical survival signals with new drugs.

**Parallel Path of the Prostate**

Charles Huggins (Figure 3) resurrected the use of endocrine ablation for the treatment hormone dependent breast cancers (103). His focus, however, was the regulation of the growth of the prostate gland and the application of that knowledge for the treatment of prostate cancer (4). He received the Nobel Prize for Physiology and Medicine in 1966. The process for deciphering the molecular mechanisms of androgen action in its target tissues and prostate cancer has tended to lag behind the pathfinder

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estrogen. Nevertheless, the basic model for the regulation of nuclear hormone receptor action is consistent but the details of androgen action are distinctly different than estrogen action which in turn created novel therapeutic opportunities to stop the biosynthesis of each active steroidal agent. The similarities and differences in the molecular actions of estrogen and androgen action are illustrated in Figure 8. The two significant differences (yet similarities) in the biosynthetic pathways between estrogens and androgens are: 1) the aromatization of the A ring of testosterone to create the high affinity ER binding ligand 17βestradiol in women. This bioactivation led to the development of aromatase inhibitors to block estrogen synthesis (51); and 2) the reduction of testosterone to the high afffinity AR binding ligand dihydotestosterone in men. This knowledge led to the development of the 5α reductase inhibitor finesteride (Figure 4) that was tested successfully for risk reduction for prostate cancer in men (104). Unfortunately, as yet, finesteride has failed to advance for use as a chemopreventive for prostate cancer because of overstated concerns about the accelerated development of potentially more aggressive prostate cancers in those men who did not have tumorigenesis prevented. In contrast, aromatase inhibitors have advanced to test their worth as chemopreventive agents (54).

A range of antiandrogenic drugs that competitively block the AR are available in clinical practice (105). Drug resistance to antiandrogen therapy parallels antiestrogen drug resistance (106) and following long term antihormonal therapy with antiandrogens, androgen induces apoptosis in antiandrogen resistant prostate cancer cells (107). Recent research has identified high local levels of androgen prediction as a major form of antihormonal drug resistance (108). As a result, a new therapeutic approach is the development of an inhibitor of androgens biosynthesis from cholesterol (Figure 8) by

blocking 17 hydroxylase/17,20 lyase (CYP17). A promising compound abiraterone acetate (Figure 4) is currently being evaluated in clinical trials (109). However, there is also a need to co-administer glucocorticoids so long term therapy must be monitored carefully.

**The successful evolution of targeted antihormonal therapy in the 20th Century and beyond**

The identification of the ER and subsequently the AR as the conduit for hormone-mediated development and growth in breast and prostate cancer respectively has had a profound effect on the approach to the treatment and prevention of cancers. These hormone mediating molecules have proved to be the pathfinders for the development of targeted therapies that transformed the approach to cancer treatment away from the nonspecific cytotoxic chemotherapy approach during the 1950's to 1990's. As a result today there is current enthusiasm for the promise of individualized medicine and tumor specific therapeutic (25, 110).

The impact of antihormonal therapy for breast cancer has been profound with improvements in patient survival, a menu of medicines now available to suit individual patient needs and a decrease in National mortality rates in numerous countries (48). Additionally, there are now two SERMS (tamoxifen and raloxifene) available to reduce the incidence of breast cancer (68, 83). But progress in our understanding and application of SERMs is more than chemoprevention. The SERM concept (71) has spread to develop tissue selective drugs for all members of the hormone receptor superfamily (25, 111). An enormous interest in developing selective glucocorticoid receptor modulators, selective progesterone receptor modulators, selective androgen receptor modulators, and

even agents to treat rheumatoid arthritis is an ongoing therapeutic outcome of translational research for the chemoprevention of breast cancer.

**Acknowledgements:**

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**Figure 1**     Timeline of the major landmarks in estrogen action and its application for the treatment and prevention of breast cancer.

**Figure 2**     The structures of estrogens, antiestrogens and SERMs mentioned in the text. The position 6 and 7 on the estradiol molecule indicate where tritium atoms were inserted to first describe estrogen binding to target tissue (26). The metabolite 4-hydroxytamoxifen (112) is an active metabolite of tamoxifen that has been the standard laboratory antiestrogen and crystallized with the ligand binding domain of the ER (96).

**Figure 3**     Professor Charles Huggins (left) and Elwood Jensen were to receive the Nobel Prize for Physiology and Medicine (1966) and the Lasker Award for their work on androgen action in cancer and the role of ER in physiology and cancer respectively.

**Figure 4**     Structures of inhibitors of estrogen and androgen biosynthesis.

**Figure 5**     Molecular mechanisms of estrogens, antiestrogens and SERMs in estrogen target tissues. The nuclear receptor complex (NRC) that results from ligand binding to either ERα or ERβ can interact with either coactivators (CoA) or corepressors (CoR) to initiate estrogenic or antiestrogenic responses respectively. The activation of transcription at a promoter site of an estrogen responsive gene or curves through the binding of the complex that is cyclically destroyed through the proteosome and then a new complex is reassembled.

**Figure 6**     A comparison of the percent homology of the domains of estrogen receptors alpha and beta abbreviations: DNA binding domain (DBD), ligand binding domain (LBD), activating functions (AF).

**Figure 7**     The evolution of resistance to selective estrogen receptor modulators (SERMS: tamoxifen or raloxifene) long term therapy.

**Figure 8**     Comparison of the molecular mechanisms of estrogens and androgens in their respective target tissues. The transformations of the respective steroids are necessary for high binding affinity for their receptors, but the activation from prohormones occurs in different tissue sites relative to their target.

Selective Estrogen Receptor Modulators and Phytoestrogens

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**ABSTRACT**

Scientific achievements in the last two decades have revolutionized the treatment and prevention of breast cancer. This is mainly because of targeted therapies and a better understanding of the relationship between estrogen, its receptor, and breast cancer. One of these discoveries is the use of synthetic selective estrogen modulators (SERMs) such as tamoxifen in the treatment strategy for estrogen receptor (ER) positive breast cancer. Hundreds of thousands of lives have been saved because of this advance. Not only is tamoxifen used in the treatment strategy for patients who have breast cancer, but also for prevention in high risk premenopausal women. Another synthetic SERM, raloxifene, which was initially used to prevent osteoporosis, is also as effective as tamoxifen for prevention in high risk postmenopausal women. In certain regions of the world, particularly in Asia, a low incidence of breast cancer has been observed. These women have diets that are high in soy and low in fat, unlike the Western diet. Interest in the protective effects of soy derivatives has led to the research of phytoestrogens and metabolites of soy that are described by some as natural SERMs. As a result, many clinical questions have been raised as to whether phytoestrogens, which are also found in other natural foods, can protect against breast cancer. This article reviews the development and role of the more common SERMs, tamoxifen and raloxifene. In addition, this paper will also highlight the emerging studies on phytoestrogens and their similarity and dissimilarity to SERMs.

**Introduction**

Great strides have been made in the last 25 years in the fight against breast cancer. One of the more notable developments has been the search for ways to prevent cancer. The development of Selective Estrogen Receptor Modulators (SERMs) has been a significant step towards achieving that goal. Tamoxifen, an antiestrogen in the breast and the pioneering SERM, has been the gold standard, and often the only choice in many countries for the treatment of breast cancer [1]. It also became the first drug ever to be approved by the United States (US) Food and Drug Administration (FDA) for the chemoprevention of breast cancer in high risk women [1]. This chapter will review the development of tamoxifen the prototypical SERM and its use and development as a chemopreventive agent. In addition this article will also highlight the emerging information regarding phytoestrogens that are being regarded by some as natural SERMs.

**Background**

By the turn of the 20th century it was known that oophorectomy in pre-menopausal women with metastatic breast cancer could cause regression of the disease [2] [3]. This showed a link between products produced by the ovaries and the growth of some breast cancers. The product was found to be estrogen [4]. In 1936, Professor Antoine Lascassagne hypothesized that breast cancer was caused by a special hereditary sensitivity to estrogen and suggested that the development of an estrogen antagonist could prevent disease [5]. Over twenty five years later in 1962 Jensen and Jacobsen [6] described the estrogen receptor (ER) as the mediator of estrogen action, setting the stage for the manipulation of this receptor for multiple purposes [7].

Investigation of possible contraceptive agents led to the reinvention of ICI 46474 a failed contraceptive agent to become tamoxifen, the first targeted anti-cancer agent. The study of tamoxifen in the laboratory led to the finding that it inhibited the growth of ER positive breast cancer cells in vitro [8]. In addition, animal studies showed that tamoxifen prevented rat mammary carcinogenesis [9] [10] but had a stimulatory effect on rat uterine weight [11]. The actions of nonsteroidal antiestrogens were clearly not wholly explainable as estrogen agonists or antagonists and a model to describe their unique actions led to the development of the SERM concept [12] [13] [14].

**What are SERMs?**

SERMs are synthetic non-steroidal agents that bind to the ER and produce a change in the biologic activity of the receptor depending on the tissue type. The primary target site for SERMs, the ER, is a nuclear receptor. To fully understand the unique nature of SERMs the actions of estrogen on the body must be revisited. Estrogen in premenopausal women is primarily produced by the ovaries. There are multiple target sites for estrogen and it has various actions throughout the body. Estrogens decrease cholesterol levels by lowering the circulating low-density lipoproteins (LDL). Its actions also include maintenance of bone density in postmenopausal women, and hormonal regulation, and control of the menstrual cycle in premenopausal women. These actions are summarized in figure 1. In contrast the effect of SERMs depends on the target sites and is shown in figure 2.

A pure estrogen agonist would be one that stimulates the positive action of estrogen at all its targets. Conversely, a pure antagonist would inhibit all the actions of estrogen at all of its target sites. In contrast, SERMs have partial agonist and antagonist properties depending on the target site hence their uniqueness. Studies have shown that the partial agonist/antagonist properties depend on which associated coregulators are expressed when the receptor ligand interaction occurs [15]. The details of the receptor/ligand interaction help us understand the mechanism of action of SERMs.

**Mechanism of Action**

There are two aspects to the mechanism of action of SERMs: the pharmacokinetics or how the drug gets to the target site and the pharmacodynamics or what it does when it gets there. Tamoxifen, (figure 3), is a lipophilic prodrug that is easily absorbed by the gut without modification and 98% is bound to albumin after entering the circulation. It undergoes extensive metabolism in the gastrointestinal (GI) tract and in the liver into its less active form N-desmethyltamoxifen and two most active forms, 4-hydroxy tamoxifen and endoxifen [16] [17] [18] [19]. Each of the hydroxylated metabolites result from first pass metabolism in the liver. These compounds enter the bloodstream via the entero-hepatic circulation to reach their target sites [18] [20] [21]. The metabolites of tamoxifen are excreted via the fecal route as has been shown by animal studies using ^{14}C radiolabeled tamoxifen [22]. These studies demonstrate 67 % of these metabolites enter the enterohepatic circulation and undergo further metabolism several times until excretion by the GI tract [23] [24]. 4-Hydroxy tamoxifen, and endoxifen have the same affinity for the ER as estrogen. Other metabolites of tamoxifen do not have as much effect or affinity for the ER as they lack the 4-hydroxy group [18]. Recent studies demonstrate that the

potent tamoxifen metabolite endoxifen is produced by the product of the CY2PD6 gene. In patients with mutations of the CYP2D6 gene or patients who take other medications that compete for the enzyme product, metabolism of tamoxifen to the potent metabolite endoxifen is affected and may therefore have less benefit [25] [26]. Raloxifene (figure 3), another SERM, is a polyphenol, which undergoes rapid conjugation in the GI tract and in the liver. In addition it also undergoes phase 3 metabolism by gut flora. The bacteria directly glucuronidate and sulfate this compound so that it is excreted [26] [27]. Since the drug does not reenter the enterohepatic circulation, it does not reach its targets as efficiently as tamoxifen. Also, a smaller percent enters the circulation as only 2% is bound to albumin and the half-life of raloxifene is 27 hours [28]. As a result of differences in metabolism and bioavailability, raloxifene is not as useful an agent in patients who already have breast cancer [29].

There are two isoforms of the ER, ER$\alpha$ and ER$\beta$ [6] [30] whose distribution and density varies depending on the target site. Both isoforms are found in the reproductive organs. Tamoxifen binds both receptors with equivalent affinity [31]. Endoxifen and 4-hydroxytamoxifen have similar affinities for both isoforms [32] and create similar gene expression profiles. Other ligands show preference for one isoform or the other, which may explain specific target tissue responses with various compounds. In many tissues, ER$\beta$ has anti-proliferative effects, whereas, ER$\alpha$ has proliferative effects [33]. Studies indicate that ER-$\beta$ has an inhibitory effect on ER-$\alpha$ [34] [36] [35]. However, the biology is more complex than a simple agonist/antagonist interaction between the two receptors. The ratio of ER$\alpha$ to ER$\beta$ at a target site may be important in determining the overall action of a SERM on that tissue. A high ratio may correlate with high levels of cellular proliferation while a low ratio implies the opposite [36].

In the past, the interaction between SERMs and the ER was thought to be a simple case of a ligand switching its target receptor on or off. Through further research it is now known that this interaction is a more complex and dynamic process. Studies using phage display created a fingerprint of exposed surfaces when tamoxifen or estrogen was bound to the ER. Different conformational changes occur in the ER depending on the ligand that binds to the ER. In addition, the fingerprint was different in ER-$\alpha$ vs. ER-$\beta$ when they were bound to identical ligands [37]. The discovery of the steroid receptor co-activator protein (SRC1) helped further to elucidate this complex interaction [40]. The binding of a SERM to the ER results in a conformational change in the ER [41], which results in the exposure of different amino acids on the receptor and the binding of different coactivators. Since the discovery of SRC1, dozens

of other co-activator and co-repressor molecules have been discovered; all of which play some role in receptor modulation [15].

Finally, another dimension of signaling pathways can modulate the ER. Activation of the ER by other growth factor pathways can result in resistance to SERMs in a tumor.

This recruitment of specific co-regulators to the ligand receptor complex depends on the ligand that binds to the ER, the ER isoform, and "cross talk" with other growth factor pathways [38]. SRC-3 is known to be important as a co-activator in breast cancer. In tumors and cancer cell lines that are HER2 positive and resistant to endocrine therapy with tamoxifen, studies demonstrate that SRC-3 is recruited to ER-α, but not ER-β in the presence of tamoxifen. In specimens from patients who were HER-2 negative and sensitive to endocrine therapy with tamoxifen, estrogen recruited SRC-3 to both ER isoforms, but tamoxifen did not [42]. Finally, when SRC-3 was knocked down, there was reduced expression of the estrogen target gene, pS2 in MCF7 cells. After the SRC-3 knockdown in cells derived from HER2 positive tumors, there was a decrease in cell proliferation and the cells regressed in the presence of tamoxifen [42].

To summarize the molecular process thus far. Once a SERM binds to the ER it causes a change in the shape of the ER. This change of shape allows recruitment of co-activators, if it is destined to elicit an estrogenic response, or co-repressors if its response is anti-estrogenic. The binding of the coregulatory molecules leads to the activation of the promoter sequence of the estrogenic responsive gene [36]. This process is also controlled by the degradation and disassembly of complexes at the gene promoter site, which causes renewed activation of the signal to initiate RNA synthesis. In this way the SERM can specifically modulate the estrogen responsiveness of a target tissue (See review Jordan Nature Reviews Cancer 2007).


**Clinical Relevance**

The full details of the mechanism of action of SERMs have yet to be precisely described however, their clinical importance as an advance in medicine is proven. Tamoxifen was initially tested in humans in the early 1970's, before extensive anti-tumor testing in animals [39] [40]. Animal testing [1] [9] [10] refocused efforts and targeted the ER [41], thereby opening the door for chemoprevention. Through animal studies tamoxifen was found to have targeted anti-tumor activity and initially, anti-estrogenic activity correlated with anti-tumor activity. These findings led to extensive human trials that helped consolidate the actions of SERMs and refined their applications. In initial human studies tamoxifen, an "antiestrogen", was found to lower bone density in pre-menopausal women [42]. However, the "estrogen-like" actions of tamoxifen, maintained bone density in post-menopausal women [43] [44]. In the uterus tamoxifen acts as an

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agonist and increases the risk of endometrial cancer in post-menopausal women [45]. The next sections review the large scale human chemoprevention trials of SERMs.

**Chemoprevention**

The first large human trial involving tamoxifen was the Royal Marsden study performed by Powles and colleagues [46] [47]. For this study approximately 3000 high-risk women were recruited and randomized to receive treatment with tamoxifen 20mg/day for 8 years or placebo. High risk status was determined by family history and a history of benign breast disease. The study found a decrease in LDL and loss of bone density in premenopausal women but, increased bone density in postmenopausal women and increased endometrial thickening on ultrasound study. Though this study initially showed no difference in the incidence of breast cancer, it was not powered to detect a difference in the development of breast cancer with either treatment group. Nevertheless, the twenty-year follow-up of this study does show a statistically significant reduction in the incidence of ER positive breast cancer in the tamoxifen treatment arm after the 8 years of treatment [48].

The National Surgical Adjunctive Breast and Bowel Project (NSABP) P-1 trial by Bernard Fisher and colleagues was the first major chemoprevention trial in the Unites Stated with tamoxifen [49]. Over 13,000 women were recruited for this study in multiple centers around the US and Canada. Once again high risk status was determined by family history, breast biopsy with pathologic findings of lobular carcinoma in situ or atypical ductal hyperplasia, no children, menarche by 12 and age at birth of first child of over 30. The initial results of the NSABP trial showed a 49% reduction in the risk of invasive breast cancer and a 50% reduction in the risk of non-invasive breast cancer. Tamoxifen also reduced the incidence of osteoporotic fractures. No difference was seen in the risk of myocardial infarction but there was an increased risk of deep venous thrombosis, endometrial cancer and cataracts in the tamoxifen group.

Based on these clinical trials in 1998, tamoxifen was approved by the US FDA for reduction of the risk of breast cancer in high-risk women. Despite the positive results of the NSABP P-1 trial the side effects noted in the tamoxifen group resurrected the interest in other SERMs that had similar chemopreventive profiles to tamoxifen but with a more desirable side effect profile. This has led to human trials with raloxifene an old compound, which had not been studied much since its discovery in the late 1970's [50] [51].

**Prevention of Osteoporosis**

In laboratory studies raloxifene was shown to inhibit DMBA induced rat mammary carcinoma growth [52], and development [53] however, it was not as potent as tamoxifen. More importantly, raloxifene was as effective as tamoxifen in maintaining ovariectomized rat bone density but was less estrogen like than tamoxifen in the rodent uterus [13] or in stimulating mouse endometrial tumor growth [54]. The short half-life of raloxifene makes it a difficult drug to dose nonetheless; clinical trials with raloxifene have also helped define its pharmacology. The Multiple Outcomes for Raloxifene Evaluation (MORE) trial evaluated the effects of raloxifene in postmenopausal women [55] [60]. This study was extended to eight years as the Continuing Outcomes Relative to Evista (CORE) trial [61]. The results of the MORE/CORE trials demonstrated the effectiveness of raloxifene in preventing osteoporosis. In addition, raloxifene also inhibited the development of invasive breast cancer by 65% [61]. These clinical data justified the evaluation of raloxifene against tamoxifen to reduce the risk of breast cancer in high risk postmenopausal women. The Study of Tamoxifen and Raloxifene (STAR) trial, was a phase III double-blinded study that randomized eligible postmenopausal women at a high risk for breast cancer, to receive tamoxifen 20mg daily or raloxifene 60mg daily [56]. The STAR trial demonstrated the equivalence of raloxifene and tamoxifen in reducing the incidence of invasive breast cancer. Furthermore, raloxifene had a better side effect profile with a lower incidence of endometrial cancer and hyperplasia, deep venous thromboses and cataracts. A drawback of raloxifene however was its decreased effectiveness in preventing the development of non-invasive breast cancer after two years, when compared to tamoxifen. Currently raloxifene is FDA approved for the treatment and prevention of osteoporosis, and risk reduction for breast cancer in high risk postmenopausal women.

**Extending Chemoprevention**

The development of a chemopreventive agent such as tamoxifen but which has significant side effects had led to interest in whether naturally occurring compounds have similar chemopreventive effects. Epidemiologic observations have made this question of even more seductive. While the etiology may be unclear it has been well documented that Asian women have a lower incidence of breast and colorectal than Caucasian women [57]. Asian diets in particular are high in soy foods, which are felt to be responsible for this difference. When Asian women emigrate to western countries their incidence of breast cancer approaches that of the indigenous population [58]. This phenomenon has been observed in Japanese and Caucasian women who emigrate to the United States. It has also been observed that the risk of breast cancer

in Asian Americans decreases in relation to increasing intake of soy derivatives [59].
Additionally, Chinese women who adopt a more westernized diet also appear to increase their
incidence of breast cancer. All these findings have generated an interest in soy foods and its
impact on hormone levels in the body. Phytoestrogens are the focus of current investigations.
However, it should be stressed at the outset that despite beliefs of benefits from changes in diet
and administration of supplements, there are dangers that breast cancer growth could be enhanced
rather than prevented.

**What are Phytoestrogens?**

Phytoestrogens are plant derivatives that bear a structural similarity to 17-beta
estradiol and act in a similar manner. Structures of common phytoestrogens, SERMs and 17- beta
estradiol are shown in Figure 3. The principal phytoestrogen groups are flavonoids, lignans,
coumestans and stilbenes [60] [61] [62]. Phytoestrogens are present in common foods such as
soybeans, grains, fruits and vegetables. An in-depth review of the various types of
phytoestrogens is beyond the scope of this article however, common properties of most
phytoestrogens include their metabolism by gut flora to additional derivatives with varying
estrogenic activity. Many studies have focused on isoflavones, which are a subgroup of the
flavonoids, they include but are not limited to genistein, daidzein and biochanin A. These
isoflavones have varying estrogenic activity [63] and isoflavones have been proposed as natural
SERMs. Studies show that isoflavones act as antioxidants in vitro and exert antiproliferative
activities [64] [65]. Equol (Figure 3), an estrogenic metabolite of the isoflavonoids family [66], is
produced from daidzein by the action of intestinal flora. This metabolic conversion however
occurs in only 30% of the population [67].

Lignans, the most prevalent phytoestrogens in the diet are found in whole wheat, fruits
and vegetables. Lignans are metabolized by the action of gut microflora into enterolactones and
enterodiol [60] with very weak estrogenic properties [66]. While there are many studies on
isoflavones, there are significantly fewer studies on coumestans and stilbenes. Coumestans are
potent activators of the ER signaling pathway but are not as prevalent in the diet. Resveratrol is
the most common stilbene and its use as a chemopreventive agent against breast cancer is actively
being studied in rodent models [60].   In the next section we will consider the mechanism of
action of phytoestrogens. The interaction of phytoestrogens with ERs is in some ways similar to
the SERM/ER interaction, but there are significant differences that confound biological
comparisons.

**Mechanism of Action of Phytoestrogens**

Hydroxylated SERMs in general have a higher binding affinity for both ERα and ERβ compared to phytoestrogens.  As with SERMS phytoestrogens can bind to either ERα or ERβ however, phytoestrogens appear to have a higher affinity for ERβ [68].  This affinity may be dose dependent but overall phytoestrogens have a significantly lower affinity for the ER than estradiol [69] [70]. In addition the estrogenic potency of phytoestrogens varies within the particular phytoestrogen group.  For example, within the flavonoid family genistein has greater potency than biochanin A, which has greater potency than daidzein [63]. Kuiper and colleagues [31] demonstrated that the stimulation of transcriptional activity by both subtypes of the ER vary depending on the estrogenic potency of the phytoestrogen and the further use of  reporter gene assays demonstrate that synthetic estrogens and phytoestrogens have varying affinity for the ER and for each ER isoforms [68][75].

SERMs are nonsteroidal estrogens that become antiestrogenic by virtue of their correctly positioned side chain.  However, the antiestrogen side chain is not present in phytoestrogens and this structural deficit may therefore limit their classifications as SERMs. Nevertheless, the presence of a correctly positioned phenolic ring and also the distance between the two opposing phenolic oxygens in the isoflavones structure is similar to that of 17 beta-estradiol (Figure 3). This similarity allows the isoflavones to bind to either subtype of ER, effectively displacing 17 beta-estradiol.  Studies have found that isoflavones have both agonistic and antagonistic effects, though they are strong ERβ agonists and weak ERα agonists [71]. It is this pharmacologic receptor interaction rather than competitive interaction at a single receptor site that may be responsible for some of the diverse biologic actions of phytoestrogens.  This action may explain how phytoestrogens protect against breast cancer, because ERβ inhibits mammary cell growth as well as the stimulatory effects of ERα [72]. However, there is yet another dimension of molecular action at the ER that might be important.  It is not certain whether isoflavones displace the estradiol by binding to a primary site on the ER, causing competitive binding between the isoflavones and the estradiol, or whether the isoflavones bind to a secondary site on the ER [73]. In contrast, genistein has been found to bind to the active site of ERβ [74].

Recent studies have attempted to decipher the actual role of each receptor subtype in gene activation and physiologic response.  Part of the problem in determining the physiologic actions of phytoestrogens is our ignorance of the actual role of the ERα and ERβ.  For example a study by Hertrampf and colleagues [75]. show that the osteoprotective effect of genistein is mediated

through the ER$\alpha$ dependent pathways and its effect is enhanced by physical activity  Also, the activation of ER$\beta$ may modulate ER$\alpha$ mediated physiological effects in vivo [82].

Many factors such as the ligand, dose and interaction of the ligand and receptor all influence ER molecular biology at the target site [76].

As with the SERMs, studies have shown that the recruitment of co-regulatory molecules may be important in determining the function of phytoestrogens. In particular, isoflavones appear to selectively trigger ER$\beta$ transcriptional pathways, especially transcriptional repression. This affinity for the ER$\beta$ results in the exposure of a weak activation function-2 (AF-2) on the surface of ER$\beta$, which has greater affinity for certain coregulators compared to ER$\alpha$ [72]. Phytoestrogens also have differential activity on several ER associated signaling pathways.  For example, Akt, which is normally phosphorylated secondary to activation of ER$\alpha$, is upregulated by genistein and daidzein in ER positive breast cancer cell lines, while resveratrol has an inhibitory effect of the phosphorylation of Akt [77].  In contrast, in ER negative cell lines, resveratrol and daidzein activate Akt and genistein inhibits activation of Akt [77].   This is clearly a non ER event, but whether this is cancer specific or a toxicity of studies conducted in vitro can only be resolved with studies in vivo.

Although the isoflavones have agonistic and antagonistic estrogenic effects, the phytoestrogens also induce differentiation as well as inhibit angiogenesis, cell proliferation, tyrosine kinase, and topoisomerase II; all of which will help prevent tumor growth. However, it is important to stress again that despite the fact that there have been numerous and extensive laboratory studies on the mechanisms of breast cancer chemoprevention with phytoestrogens, there is no definitive evidence that proves that phytoestrogens are chemopreventive but they may contribute to adverse outcomes in breast cancer [78].

**Cell and Animal Studies on the Effect of Phytoestrogens**
Phytoestrogens have been likened to natural SERMs, and a brief survey of cell and animal studies of phytoestrogens reveals some similarities to SERMs such as tamoxifen . The approach to these studies may be classified into three broad categories. The first are studies that focus primarily on the role of phytoestrogens as a chemopreventive agent.  The second are those

studies that focus on phytoestrogens as a treatment agent.  The third are those studies that focus on the biological effects when phytoestrogens are used continuously from neonates to adults.

The first category focuses on the chemopreventive effects of phytoestrogens in animal models that are subsequently treated with a chemical carcinogen.  Animal studies have shown that when rats are treated with phytoestrogens and then exposed to a carcinogen they are less likely to develop breast cancer if exposure to phytoestrogens occurs at an early age [79] [80]. Lamartiniere and colleagues [79] demonstrated that the timing of exposure to phytoestrogens whether pre or post puberty, may influence their action on preventing mammary carcinogenesis. Lamartiniere [79] found that neonatal injections of genistein reduced the incidence of DMBA induced mammary tumors in rats. Further evaluation revealed that the overall effect of genistein on prepubertal rats appeared to be secondary to early differentiation in mammary tissues resulting in less active EGF signaling pathways in adulthood that may be protective against breast cancer. A recent meta-analyses by Warri et al [81] revealed pubertal exposure to  phytoestrogens result in changes in the mammary gland morphology and signal pathways that mimic those induced by the estrogenic environment of early first pregnancy.

The second group of studies focus on the use of phytoestrogens treatments in both tumor implanted athymic mice and breast cancer cell lines. Studies have shown that treating estrogen sensitive MCF-7 cell lines with genistein has an inhibitory effect on their growth [82]. However, not all studies have had such conclusive findings as the action of phytoestrogens on breast cancer cells may be dose dependent. At low concentrations phytoestrogens may stimulate growth, and at high concentrations inhibit growth [66] [82] [83] [84] [85]. The studies by Helferich help elucidate the dose dependent actions of isoflavones [93] [86] . In animal studies, in which ovariectomized athymic mice were implanted with MCF-7 cells, genistein promotes the growth of ER+ MCF 7 cells and the effect of this isoflavone was dose dependent.  At concentrations as low as 10nM genistein promoted growth of ER dependent MCF-7 cells in vitro [86]. At higher concentration (> 20microM) genistein inhibited the MCF-7 cell growth. In addition genistein can stimulate growth of MCF-7 cells in vivo in a dose dependent manner [87].  Clearly, these data call for caution with the use of phytoestrogens in women with breast cancer.

Indeed, the early study by Welshons et al [66] cautioned against the use of antihormonal therapies that did not block the ER for the treatment of breast cancer because high fiber or exclusively vegetarian diets with phytoestrogens containing food supplements could enhance the probability of tumor recurrence and growth. Furthermore the combination of phytoestrogens and tamoxifen to treat breast cancer may result in decreased efficacy of tamoxifen.  In a study evaluating the development of tumor and the tumor latency period, tamoxifen treated mice fed a

low dose isoflavone enriched diet had a higher tumor incidence and a shorter tumor latency period than placebo treated mice [95].  In addition tamoxifen associated mammary tumor prevention was also significantly reduced. Nevertheless, certain phytoestrogens have also been noted to cause apoptosis of human breast cancer cells and this occurred at concentrations of 20 – 25 micromol/L [88] [89] [90]. While phytoestrogens have been observed to cause these various actions in vitro, it is unclear that in vivo the concentrations needed to achieve these actions are attainable. In animal studies a protective effect of phytoestrogens on the development of mammary cancer are conflicting [91] [92]. Santell and colleagues [92] have shown that while genistein may inhibit breast cancer cells in vitro, treatment of tumor bearing athymic mice with genistein did not inhibit tumor growth, however in their study ER negative human breast cancer cell lines were used. It would seem that the ability of phytoestrogens to be toxic in vitro at high concentrations does not extrapolate to models in vivo where the ability to maintain high local concentrations for long periods may be impaired.

A third approach is the study of the effects from early exposure to phytoestrogens from the perinatal periods and onwards.  This approach was recently adopted by Mardon and colleagues [93].  Rats perinatally or lifelong exposed to a rich isoflavone diet exhibited higher body weight and fat mass at 24 months of age.  Perinatal exposure to phytoestrogens led to higher bone mineral density in later life [93].  The translation of these data to human epidemiology and pharmacology is the challenge and has no immediate application to effects on mammary carcinogenesis.  The observation is an estrogen-like action on bone rather than SERM related.

**Human Trials**

Human trials on phytoestrogens differ from SERMs because unlike the SERMs, there are no major large-scale prospective studies of chemoprevention and pharmacology. Human studies on phytoestrogens can be divided into two broad categories.  The first are studies that evaluate the effect of phytoestrogens on estrogen biosynthesis and excretion, the second are those studies that evaluate the overall impact of dietary phytoestrogens on specific clinical endpoints such as menopausal symptoms and bone mineral density presumably through a stimulatory action through the ER.   Many studies have examined the use of phytoestrogens as chemopreventive agents however, these studies are of limited value as they are retrospective.

**Estrogen Biosynthesis and Excretion**

Human studies on the effect of phytoestrogens on estrogen biosynthesis and excretion usually evaluate levels of circulating estrogen or steroid byproducts and metabolites in the urine. In addition in many of these studies the levels of phytoestrogens are also measured and factors that affect these levels are explored. Human studies have shown conflicting results regarding the overall effect of phytoestrogens. Lu and colleagues [94] treated 10 pre-menopausal women with a soy containing diet beginning on day two of the menstrual cycle to day two of the next cycle. Blood and urine samples were obtained before and during the initiation of the soy diet. Their results showed that the circulating levels of 17-beta estradiol decreased by 25%, however, cycle length did not change [94]. A dietary intervention study by Kumar and co-workers showed similar findings [95]. This study randomized women to receive 40mg of isoflavones day or placebo for a 12-week period. They found that serum free estradiol and estrone levels decreased. Serum hormone binding globulin increased and mean cycle length also increased. Conversely, a yearlong dietary intervention study by Maskarinec and co-workers [96] in premenopausal women did not find any difference in cycle length or hormone levels. These studies raise the question that while dietary intake of phytoestrogens is important, intake alone may not be the determinant of a chemoprotective effect.

Since a Finnish case control study [97] suggests that high enterolactone concentrations are associated with decreased breast cancer risk., it is possible that lifestyle factors that affect enterolactone may be linked to breast cancer risk. Whether these lifestyle factors that control enterolactone levels are linked to breast cancer risk remains to be seen. Administration of antibiotics has been noted to decrease the serum concentration of enterolactone for a prolonged period [98]. Premenopausal women who are treated with long term antibiotics for urinary tract infections seem to be at higher risk for breast cancer, presumably because it alters the gut metabolism of phytoestrogens [99]. Smoking and obesity have been noted to decrease plasma enterolactone levels, however, tea, coffee, fiber and vegetables have the opposite effect [100]. In a study monitoring plasma enterolactone levels, women were noted to have a higher plasma concentration while on wheat bread 41.1nmol/L compared to 15.4nmol/L while on white bread [67]. Links to actual cancer risk do not exist but associations have been noted.

In human studies, it is often difficult to measure serum levels of phytoestrogens, because of a short half-life. Since most phytoestrogens are excreted in the urine, urine analysis of metabolites of phytoestrogens can be used to give an indication of exposure to phytoestrogens [101]. Urinary excretion of phytoestrogens varies in different regions of the world [102]. Women in areas with a low incidence of breast cancer have higher urinary isoflavonoids than women living in areas with a high incidence of breast cancer. Vegetarians also have a higher

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concentration of isoflavonoids in their urine than omnivores [103]. The excretion of equol in the urine has been proposed as a possible marker of the chemoprotective effect of phytoestrogens [112] [113]. Duncan and colleagues [104] studied the hormone profile of equol excretors versus equol non-excretors and found that regardless of the amount of phytoestrogens ingested in the diet, equol excretors had decreased levels of estrone, estrone-sulfate, testosterone, DHEA and higher levels of steroid hormone binding globulin. This steroid hormone profile has been found to be a protective profile for breast cancer.  The possible mechanisms to create a "change profile" may include the findings that phytoestrogens stimulate the production of sex steroid binding globulin by liver cells [103] and have inhibitory effects on the enzymes involved in the synthesis of estrogen. Phytoestrogens are known to decrease the conversion of androgens to estrogen by blocking the aromatase enzyme system. [105].

**Phytoestrogens and Clinical Endpoints**

The second group of human studies are those that focus on the effect of phytoestrogens on focal clinical endpoints.  These endpoints vary and include alleviation of menopausal symptoms, maintenance of bone mineral density and development of breast cancer in some retrospective studies.   Given recent concern regarding the possible adverse effects of hormone replacement therapy other alternatives for treatment of menopausal symptoms have been explored and phytoestrogens have played a significant role.  A recent Cochrane review of the database revealed no clear evidence of the effectiveness of phytoestrogens in alleviating menopausal symptoms [106]. This notwithstanding there are some small trials which show a benefit to using phytoestrogens for treating menopausal symptoms. In a double blind prospective study sixty women were randomized to receive 60mg of isoflavones daily for 3 months or placebo [107]. The menopausal symptoms before and after treatment were recorded.  Women receiving the phytoestrogens treatment noted a 57% and 43% decrease in the incidence of hot flashes and night sweats respectively.  Similar results were seen in a small trial using a 6 week treatment of flaxseed for the treatment of menopausal symptoms [108]. Some investigators are evaluating the use of phytoestrogens as alternative agents to hormone replacement therapy (HRT) in the management of post menopausal symptoms [107].  Recently, prenylated flavonoids derived from hops are being used to treat menopausal symptoms.  One such compound is 8-prenylnaringenin (Figure 3) that has strong estrogenic activity [109]. MenoHop an agent containing the

phytoestrogen 8-prenylnaringenin, is currently being evaluated to treat menopausal complaints in Belgium [110].

The relationship between phytoestrogens and bone health remains unclear, with some studies showing a benefit associated with phytoestrogen treatment and others showing none [111]. Supplementation of diet with isoflavones has been shown to help maintain lumbar spine bone density [122] [112]. A randomized double blind control trial was performed to compare with HRT, the effect of the phytoestrogen genistein on bone metabolism and bone mineral density [113]. Patients were randomized to receive either HRT daily (1mg of 17beta-estradiol and 0.5mg norethisterone) or genistein 30mg daily or placebo daily for a period of 1 year. On completion of this protocol women receiving the HRT and genistein had significantly increased bone mineral density in the femur compared to those in the placebo group. In another randomized control trial, Atkinson and colleagues [114] showed that women receiving an isoflavones extract had a decreased loss of lumbar spine bone mineral content and bone mineral density compared to placebo.

Direct studies on the efficacy of phytoestrogens in preventing breast cancer are difficult given the length of time required to perform such a study. Indeed, this obstacle with phytoestrogen research illustrates how powerful SERMS are to produce dramatic decreases in breast cancer incidence within 5-10 years [55] [115]. However, surrogate endpoints such as the effect of phytoestrogens on breast cell proliferation and mammographic density have been studied. Increased breast cell proliferation and increased mammographic density are risk factors for malignancy. Short-term dietary supplementation with phytoestrogens stimulates breast epithelial proliferation [116]. This finding has also been noted in premenopausal women treated with prolonged phytoestrogen intake [117]. This breast proliferation is evident on mammograms as increased mammographic densities and some of these parenchymal patterns are associated with a higher risk of breast cancer [118]. These histological findings are supported by the observation of increased high risk parenchymal sonographic patterns in women who report low dietary soy protein intake [119]. Other studies such as that by Maskarinec and colleagues [120] show a similar finding in mammographic density in women treated with prolonged phytoestrogen supplementation.

As noted in animal studies, [101] the age at which a woman is exposed to phytoestrogens and length of exposure to phytoestrogens may be important in determining whether a protective benefit is obtained. A prospective 12 year study of diet and breast cancer by Key and colleagues [121] of over 30,000 women in Japan showed there was no relationship found between soy food consumption and the development of breast cancer, however this study

was comprised of mostly non-adolescent women. In contrast, Shu and colleagues [122] performed a retrospective case controlled study on Chinese women with breast cancer. Subjects completed a questionnaire regarding their dietary intake in adolescence. A high soy consumption as an adolescent was associated with a decreased incidence of breast cancer as an adult. This may also explain why when women emigrate to countries with a higher incidence of breast cancer than their native country, they are more likely to have a decreased incidence of breast cancer if they emigrated after puberty [123].

While there is increasing excitement at the possible role of phytoestrogens as chemopreventive agents or as complimentary alternative medicine for menopausal symptoms their safety profile remains largely unknown and concerns regarding this have been raised in two recent reviews [124] [125]. Isoflavones such as genistein have been found to stimulate the growth of MCF-7 cells [86] [93]. Some studies have shown that soy products increase breast epithelial cell proliferation [125] [126], which may increase the risk of breast cancer. These findings suggest caution in the broad use of phytoestrogens. In addition the interaction of phytoestrogens and tamoxifen is breast cancer patients may negate the protective effects of SERMs and caution have been advised against combination of these two agents [126].

**Conclusion**

Since their discovery the use of SERMs in clinical practice continues to expand [127] [128] [129]. As our knowledge of phytoestrogens grows, so does our understanding of its interaction with the ER and its ability to possibly act as a natural SERM or conversely to antagonize the actions of SERMs. However, based on their structure function relationships, the molecular endocrinology of SERMs and phytoestrogens is very different and the phytoestrogens appear to act as ER agonists at low concentrations but may act as antagonists by biochemical mechanisms through the ER beta receptor complex. Despite the advances in the treatment of breast cancer, prevention if possible must be superior to treatment. Currently tamoxifen and raloxifene are the first important steps in the quest to develop a complete preventative agent. In the future, a role, if any for the phytoestrogens or their derivatives may emerge, but current research is too weak to provide any clinical guidelines beyond caution. Alternatively, clues from laboratory studies may prove to be important in future drug development. An example of this is the current interest in the pharmacology of resveratrol which may have valuable pharmacological actions not mediated via the ER. [130] [131].

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Figure Legend:


Figure 1:  The sites of action for estrogen.

Figure 2: The sites of action of tamoxifen.

Figure 3:  A structural comparison of commonly studied phytoestrogens and pphytoestrogen
metabolites to SERMs

# Figure 1

## Estrogen Target Tissues



Hypothalamo Pituitary Axis

Gonadotropins    Sex Steroids

Liver (Cholesterol and Lipids)

Some Breast Cancers

Uterus

Vagina

Bones
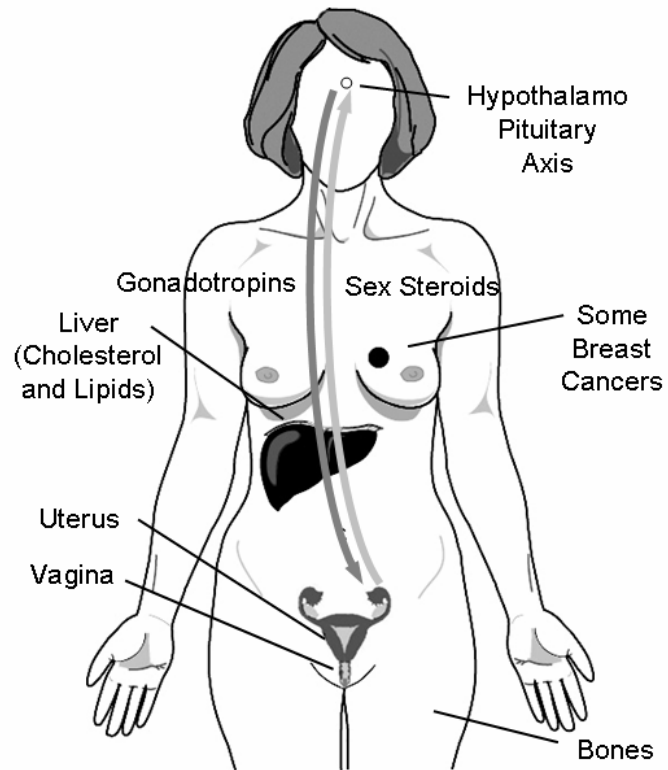
**Figure 2**



Selective Action of Tamoxifen

Inhibits Second Primary Breast Cancer

Lowers Circulating Cholesterol

Preserves Lumbar Spine Bone Density

Inhibits Recurrence

Increases Endometrial Thickness and Cancer
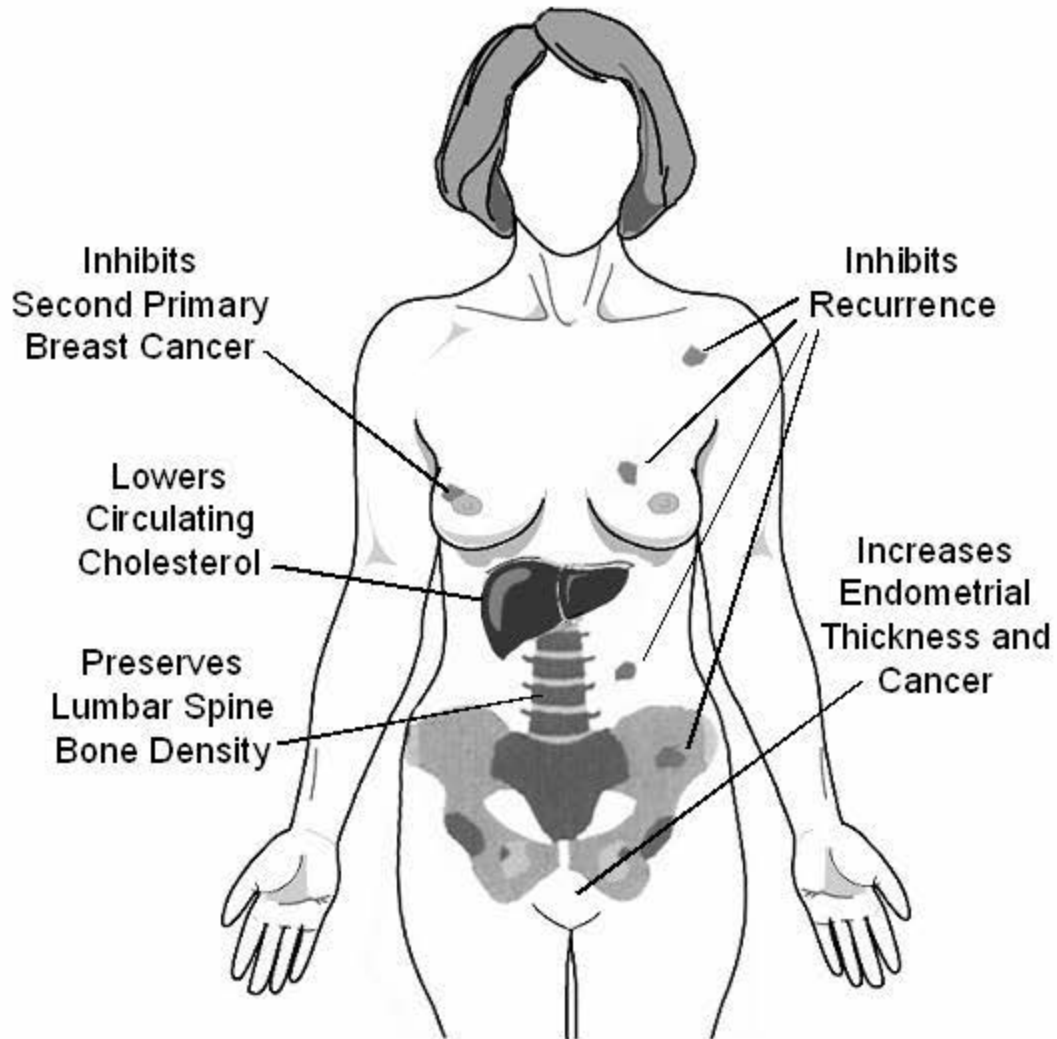
**Figure 3:**
Structures of Estradiol, SERMs and Phytoestrogens

Isoflavones:
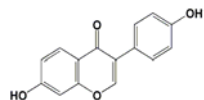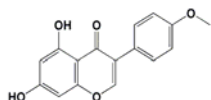


Daidzein          Biochanin A          Genistein          Equol          8-Prenylnaringenin
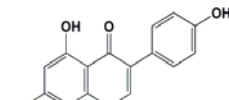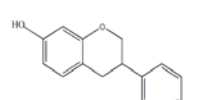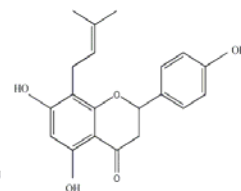
Lignans:                                    Coumestans:          Stilbenes:
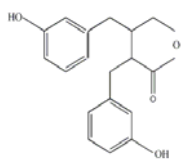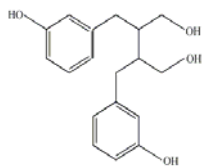


Enterolactone          Enterodiol          Coumestrol          Resveratrol
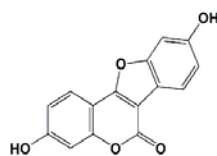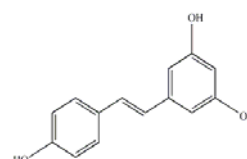
Estrogens:          SERMs:
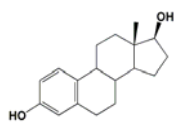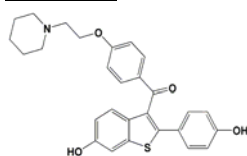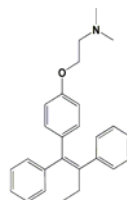


Estradiol                Raloxifene          Tamoxifen

1 **Tyrosine phosphorylation of the nuclear receptor coactivator AIB1/SRC-3 is**

2 **enhanced by Abl kinase and is required for its activity in cancer cells.**

3

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7

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17

18 Running title: Tyrosine phosphorylation site on coactivator AIB1/SRC-3

19

22

1     Abbreviations: AIB1, amplified in breast cancer 1; SRC-3, steroid receptor coactivator-3;

2     p/CIP, CBP interacting protein; CBP/p300, CREB binding protein; IGF, insulin-like

3     growth factor; EGF, epidermal growth factor; HER2/neu, v-erb-b2 erythroblastic

4     leukemia viral oncogene homolog 2; ERα, estrogen receptor alpha; PR-B, progesterone

5     receptor; c-Abl, ABL1, v-abl Abelson murine leukemia viral oncogene homolog 1;

6     CARM1, coactivator-associated arginine methyltransferase 1; MALDI-TOF, matrix

7     assisted laser desorption/ionization – time of flight; MMTV, mouse mammary tumor

8     virus.

1 **ABSTRACT**

2 Overexpression and activation of the steroid receptor coactivator AIB1/SRC-3 has been

3 shown to have a critical role in oncogenesis; required for both steroid and growth factor

4 signaling in epithelial tumors. Here, we report a new mechanism for activation of SRC

5 coactivators. We demonstrate regulated tyrosine phosphorylation of AIB1/SRC-3 at a C-

6 terminal tyrosine residue (Y1357) that is phosphorylated after IGF-1, EGF or estrogen

7 treatment of breast cancer cells. Phosphorylated Y1357 is increased in HER2/neu

8 mammary tumor epithelia and is required to modulate AIB1/SRC-3 coactivation of ERα,

9 PR-B, NF-kB and AP-1 dependent promoters. c-Abl tyrosine kinase directly

10 phosphorylates AIB1/SRC-3 at Y1357 and modulates the association of AIB1 with c-

11 Abl, ERα, the transcriptional cofactor p300, and the methyltransferase CARM1.

12 AIB1/SRC-3 dependent transcription and phenotypic changes, such as cell growth and

13 focus formation, can be reversed by an Abl kinase inhibitor, imatinib. Thus, the

14 phosphorylation state of Y1357 can function as a molecular on/off switch and facilitates

15 the cross-talk between hormone, growth factor and intracellular kinase signaling

16 pathways in cancer.

17

18

19

3

1 **INTRODUCTION**

2 Coactivators significantly enhance the rate of transcription by binding to, and

3 bringing together, components of the basal transcriptional machinery complex at gene

4 promoters. A member of the p160 steroid receptor coactivator (SRC) gene family, AIB1

5 (also called SRC-3;TRAM1; RAC3; ACTR; NCOA3) is amplified and its corresponding

6 mRNA and protein levels are overexpressed in multiple cancers (3, 20, 29, 43, 58).

7 Overexpression of AIB1/SRC-3 is associated with markers of poor prognosis in breast

8 cancer cells, including increased p53 expression, being HER2 positive and lacking ER

9 and PR expression (5, 38). Phenotypic studies strongly argue that AIB1/SRC-3 has a role

10 in both hormone- and growth factor- dependent gene expression. Cancer cell line studies

11 demonstrate that AIB1 is critical for estrogen (28) and IGF-1 dependent growth; it

12 protects cells against apoptosis or anoikis (37) and increases cell size and proliferation

13 (64). AIB1 also regulates EGFR tyrosine phosphorylation and the subsequent

14 downstream EGF induced activation of STAT5 and c-Jun N-terminal kinase (25).

15 Targeted disruption of p/CIP, the mouse homologue of AIB1, demonstrates that AIB1 is

16 critical for somatic growth (54, 59), energy balance (53), adipogenesis (30) and the rate

17 of oncogene (24) and carcinogen-induced tumor formation (23). Overexpression of AIB1,

18 or its naturally occurring isoform AIB1-Δ3 in mice, caused increased mammary gland

19 size, increased mammary epithelial cell proliferation (50) and increased tumor incidence

20 in multiple organs (51).

21 Site-specific phosphorylation and dephosphorylation is a common post-translational

22 modification utilized to control target protein functions. For AIB1, serine and threonine

23 phosphorylation has been described (57) and can be an initiating modification that occurs

1  before further post-translation modifications, e.g. sumoylation (55), ubiquitylation (16,

2  32, 56) or methylation (13, 33). How tyrosine phosphorylation regulates the interactions

3  of AIB1 with these other modifying enzymes or with other transcription co-factors and its

4  relationship to pathway signaling is examined here for the first time. Our study

5  documents that a single, site-specific AIB1 phosphorylation (at Y1357) can change the

6  interaction of AIB1 with three proteins often found in transcription complexes bound to

7  promoter elements: a methyltransferase (CARM1), a histone acetyl-transferase (p300)

8  and a nuclear receptor (ERα). Dynamic simulations suggest a molecular mechanism for

9  these changed interactions post-phosphorylation. For the first time, we demonstrate a

10 novel role for c-Abl (Abl) kinase in steroid receptor signaling via alteration of coactivator

11 function. Abl kinase directly phosphorylated and bound to AIB1 via the Y1357 site.

12 These results suggest an on/off switch for coactivating ability and that cross-talk between

13 steroid and growth factor signaling can occur in breast cancer cells via modulation of

14 AIB1 Y1357 phosphorylation. Furthermore, detection of phospho-Y1357 is potentially a

15 response marker in cancer tissues for inhibitors of Abl, such as imatinib (Gleevec[®]).

16

17 **MATERIALS AND METHODS**

18 *Plasmids and Reagents:* p300-HA, CARM1-HA, and c-Abl-AU5 plasmids were kindly

19 provided by Maria L. Avantaggiati (Georgetown University), Michael .R. Stallcup

20 (University of Southern California), and J. Silvio Gutkind (NIH/NIDCR). AIB1-Δ3

21 plasmid was previously described (42). AIB1-Δ3-FLAG tag expression plasmids (WT,

22 Y1357F, and S505A constructs) were made by PCR amplification of ACTR/AIB1-Δ3

23 cDNA (778 bp to 4422 bp) to add new 5' Not I and a 3' Bgl II sites. PCR product was

cloned into p3XFLAG-CMV-10 (Sigma-Aldrich, Inc). Imatinib (STI-571, Gleevec®; Novartis, Inc.) was kindly provided by Jeffery A. Toretsky (Georgetown University). EGF was purchased from Roche Diagnostics Co. IGF-1 was purchased from R&D Systems.

*Cell lines:* MCF-7 and COS-7 cells were grown in IMEM media. (Invitrogen Co.) with 10% heat inactivated fetal bovine serum (HI-FBS, Quality Biological Inc.). MDA-MB-231, A549, HeLa and 293T cells were grown in DMEM (Invitrogen Co.) with 10% HI-FBS. CHO-K1 cells were grown in F12-DMEM (Invitrogen Co.) with 10% HI-FBS. Cells were hormone stripped in 5% charcoal/dextran stripped FBS (CCS; Hyclone) containing media.

*Immunoprecipitation and western blot analysis: IP experiments with MCF-7, A549 and MDA-MB-231 cells*: cells were grown to 80% confluency in 150 mm dishes, serum starved for 24 hours, treated -/+ 50 ng/ml of IGF-1 or EGF for 10 min. Cells were washed cold PBS pH 7.4 and harvested with 1% NP-40 lysis buffer + 1 mM $NaO_3VO_4$ + 1X Complete Protease Inhibitor cocktail (Roche Diagnostics Co.). *IP experiments with 293T cells*: 293T cells were transfected with 4 μg of each plasmid. Antibodies used for IP: 4G10 pY Ab agarose conjugate (Upstate Biotech, Inc.); AIB1 mAb (BD Transduction Labs); phospho-Y1357 AIB1 polyclonal Ab (Pacific Immunology Co.); FLAG M2 affinity gel (Sigma-Aldrich, Inc.); HA affinity Matrix (Roche Diagnostics); AU5 (Covance Co.); Abl (BD Biosciences); ERα Ab-7 (Lab Vision Co.). IP was performed as previously described (25). Protein lysates were subject to NuPAGE gel electrophoresis

1 (Invitrogen Co.). *Western blot analysis (WB):* WB analysis was done as previously

2 described (37). Additional antibodies used for WB: phospho-CrkL Y207 (Cell Signaling

3 Co.); ERα Ab-15 (Lab Vision Co.); Actin (Millipore Co.); HA (Roche Diagnostics Co).

4

5 *Phosphorylation mapping. Sample preparation.* Serum starved MCF-7 cells were treated

6 for 10 min with 50 ng/ml IGF-1 or EGF (R&D Systems). Whole cell lysates were

7 harvested with 1% NP-40 lysis buffer, pre-cleared, immunoprecipitated with anti-AIB1

8 mAb (BD Transduction Labs) and run on a 4-12% SDS-PAGE gel (Invitrogen Co.).

9 Phosphorylation mapping by ProtTech Inc.: Sequence grade modified trypsin (Promega

10 Co.) or Asp-N (Roche Diagnositics) was used for protein digestion reactions. For each

11 digest, ~20-50% of the sample was used for phosphatase differential analysis. Two

12 aliquots of peptide mixture were analyzed for each digestion: to the treated reaction, one

13 unit of alkaline phosphatase (Roche Diagnostics) was added, while in the control reaction

14 heat-inactivated alkaline phosphatase was used. Both samples were commercially

15 analyzed by MALDI-TOF MS (Micromass Proteome Work System MALDI-TOF

16 Reflectron mass spectrometer). α-cyano-4hydroxycinnamic acid was used as a matrix.

17 Phosphopeptides were identified by manually comparing spectrum from phosphatase

18 treated and control samples.

19

20 *Luciferase reporter assays.* Luciferase assays were performed as previously described

21 (42) using Luciferase Assay System (Promega Co.). $3x10^4$ of hormone stripped cells

22 were plated in each well of a 24 well plate. Cells were transfected with FuGENE (Roche

23 Diagnostics) for 16-24 hrs and then treated with hormones for 24 hrs. Cell extracts were

1   prepared by using 100 μl of 1X Passive Lysis Buffer (Promega Co.) and incubating at

2   room temperature for 30 min on a rocker. 20 μl of the cell extract was assayed for firefly

3   luciferase activity with the Luciferase reporter assay kit (Promega Co.). Protein

4   concentrations for each sample were determined using Bradford protein assay.

5   Luciferase values for each sample were normalized with their protein concentration.

6

7   *Reverse-transcription real time PCR.* MCF-7 cells were transfected with AIB1 (3 ug) and

8   ERα (0.5 ug) (AMAXA kit V, program E-14) for 24 hrs. Cells were estrogen stripped

9   and treated with E2 100 nM for 3 hrs and total RNA was harvested using RNA STAT

10  (Tel-Test Inc.) 150 ng of RNA was used to perform reverse-transcription real time PCR

11  with the Platinum Quantitative RT-PCR Thermoscript One-Step System (Invitrogen).

12  Samples were reversed transcribed for 30 min at 56°C followed by 3 min 95°C

13  denaturing step and 40 cycles of 15 sec 95°C and 1 min 58°C. Fluorescence data was

14  collected during the 58°C step (iCycler, Biorad). pS2 (TFF-1) probe and primers were

15  purchased from Applied Biosystems (cat no. Hs00170216_m1); beta-actin primers and

16  probe: forward - 5' cct ggc acc cag cac aat, reverse - 5' gcc gat cca cac gga gta ct, probe -

17  5' FAM/ tca aga tca ttg ctc ctc ctg agc /BHQ (IDT DNA  Inc).

18

19  *Site directed mutagenesis.* The QuikChange XL II Mutagenesis kit (Stratagene Co.) was

20  used to introduce amino acid mutations in pCDNA3-AIB1-Δ3 and pCMV-3XFLAG-

21  AIB1-Δ3. The following primers (IDT Inc.) were used for the mutagenesis reaction:

22  **Y1357F**: sense, 5'phos – ccg cag gct gca tcc atc ttc cag tcc tca gaa atg aag gg; anti-sense,

23  5'phos - ccc ttc att tgt gag gac tgg aag atg gat gca gcc tgc gg. The mutagenesis reaction

1     was performed under the following conditions using the RoboCycler 40. PCR reaction

2     conditions: 5 µl 10X QuikChange reaction buffer; pCDNA3-AIB1-Δ3 (200 ng); sense

3     primer (100 ng); anti-sense primer (100 ng); 1 µl of dNTP mix; 3 µl Quik solution;

4     brought up to a volume of 50 µl. PCR cycling conditions: step 1: 95°C for 2 min for 1

5     cycle; step 2: 95°C for 1 min, 60°C for 1 min, 68°C for 30 min for 25 cycles; step 3:

6     68°C for 7 min. The DNA from the mutagenesis reaction was digested with 1 µl of Dpn I

7     restriction enzyme for 1 hr at 37°C to digest template DNA. 4 µl of the digested reaction

8     was transformed into 45 µl of β-mercaptoethanol treated XL-10 gold competent cells.

9     Plasmid DNA was prepared and DNA sequencing was performed to confirm mutation.

10

11     *Phospho-antibody production.* A rabbit polyclonal antibody to phospho-Y1357 AIB1 was

12     raised against the phosphorylated peptide NH2-SIpYQSSEMKGWPSGNLC-COOH

13     (Pacific Immunology Co.). Titers against the phosphorylated and non-phosphorylated

14     peptides were confirmed by ELISA. Phospho-specific antibodies were purified

15     sequentially using non-phosphorylated and then phosphorylated peptide affinity columns.

16

17     *Immunohistochemistry (IHC).* AIB1/SRC-3-/- (p/CIP-/-) transgenic mice were previously

18     described (59). FVB/N-TgN (MMTV-HER2/neu) mice were purchased from Jackson

19     Laboratories. IHC analyses were performed on mammary gland #4 and tumor sections as

20     previously described (50) using the phospho-Y1357 AIB1 rabbit polyclonal Ab.  Briefly,

21     tissues were fixed in 10% formalin and blocked in paraffin.  4 µm paraffin embedded

22     sections of mammary gland and tumor tissue were deparaffinized in xylene, rehydrated in

23     alcohol, boiled for 10 minutes in citrate buffer (pH 6) (Zymed Labs) for antigen retrieval

1    and quenched with 3% hydrogen peroxide. The primary antibody was incubated

2    overnight at 4°C. The phospho-Y1357 blocking peptide (Genscript) was prepared at 4

3    times the concentration of the phospho-Y1357 antibody.  The peptide and antibody

4    solutions were incubated together for 30 minutes at room temperature.  The entire volume

5    was added to the tissue section and incubated overnight at 4C. Detection rabbit primary

6    antibodies were performed using the DAKO Envision Plus HRP kit (DAKO

7    Cytomation). Bound antibody was visualized using DAB substrate (Vector Labs).  The

8    slides were countered stained with hematoxylin (Polysciences, Inc.) for 30 seconds,

9    dehydrated through an ascending concentration of ethanol, cleared in xylene and mounted

10    with Clearmount Solution (Zymed Labs).

11

12    *Protein modeling. Structure prediction:* 3D models of Y1357 were generated based on

13    BLAST sequence alignment (1) with available crystal structures: 1SR9 (PDB

14    annotation). Structure predictions for Y1357 were performed with the MODELLER 7v7

15    program (22). *Energy Minimization and Molecular Dynamics (MD):* The predicted wild

16    type and phosphorylated structures were energy minimized using the consistent valence

17    force field (CF91) with default partial atomic charge available in Discover v3.0. 300ps

18    MD simulations with distant-dependent dielectric constants were carried out using

19    SANDER module of the AMBER7.0 suite programs (7) with PARM98 force-field

20    parameter (Accelyrs Inc).

21

22    *Abl in-vitro kinase assay.* Recombinant c-Abl kinase (80 ng) (Invitrogen Co.) was

23    incubated with GST-AIB1 1017-1420 aa (Don Chen, UMDNJ-Robert Wood Johnson

1    Medical School) purified from BL21 cell lysate. The reaction was performed for 30 min

2    at 30ºC in kinase buffer (50 mM Tris pH 7.5, 10 mM MgCl$_2$, 0.01% NP-40, 1 mM DTT,

3    0.5 mM ATP). Phosphorylation was detected by western blot with p-Y1357 AIB1 pAb.

4

5    *Cell growth assays:* Validated Abl siRNAs (exon 3 #1346; exon 11 #1431) were

6    purchased from Ambion Co. and transfected as previously described (37). Hormone

7    stripped MCF-7 cells were plated in 1% CCS and 10 nM ICI 182,780 (Tocris

8    Biosciences) with or without 10 nM estrogen. Cell growth was measured by utilizing the

9    WST-1 reagent (Roche Diagnostics) after 4 days.

10

11    *Focus formation assays:* AIB1/SRC3-/- MEFs were kindly provided by Jianming Xu

12    (Baylor College of Medicine). $2 \times 10^6$ SRC3-/- MEFs were transfected with 2 ug of H-ras

13    V12  and either 4 ug of empty vector, AIB1-Δ3  (WT) or AIB1-Δ3 Y1357F constructs

14    using the AMAXA MEF kit 2 (program A-23), plated in 100 mm dishes and grown for 3

15    weeks with regular media changes. Plates were fixed with ice cold methanol and stained

16    with crystal violet (0.5% crystal violet/ 25% methanol).

17

18    **RESULTS**

19    **Tyrosine phosphorylation of AIB1 in breast cancer cell lines.**

20    We first investigated the change in overall tyrosine phosphorylation of AIB1 in MCF-7

21    breast cancer cells that had been treated with IGF-1. These cells were used because AIB1

22    is rate-limiting for IGF-1 stimulation of their growth (37). AIB1 tyrosine phosphorylation

23    was examined by immunoprecipitation (IP) of AIB1 from whole cell extracts and

1  possible tyrosine phosphorylation of AIB1 was detected by western blot analysis with an

2  anti-phospho-tyrosine antibody (PY) (Figure 1A). IGF-1 treatment increased by 2-3 fold

3  a phospho-tyrosine containing band of MW 165 kDa, which was identified as AIB1 by

4  re-probing the blot with the AIB1 antibody (indicated with * in Figure 1A). We

5  previously demonstrated that AIB1 is critical for EGF signal transduction in the MDA-

6  MB-231 breast cancer cell line (25). Therefore, we asked if EGF treatment of this cell

7  line would also increase tyrosine phosphorylated AIB1 levels. We observed a significant

8  increase in the phospho-tyrosine AIB1 levels after 10 min of EGF stimulation (Figure

9  1B). The blot was stripped and reprobed with the AIB1 antibody to confirm that this

10  phospho-tyrosine band was AIB1. These results demonstrate that growth factor induced

11  tyrosine phosphorylation of AIB1 is not limited to a single breast cancer cell line and that

12  AIB1 can be tyrosine phosphorylated by both IGF and EGF signaling pathway kinases.

13

14  **Mapping of a phosphorylated tyrosine residue (Y1357) in AIB1.**

15  To identify specific growth factor induced tyrosine residues in AIB1, we employed the

16  mass spectrometry (MS) technique, MALDI-TOF. AIB1 in total lysates from IGF-1 and

17  EGF treated MCF-7 cells was immunoprecipitated with a monoclonal AIB1 antibody.

18  Samples were run on a SDS-PAGE gel and a band corresponding to AIB1 was excised

19  and its protein sequence confirmed by Nano LC-MS/MS technique before post-

20  translational modification analysis was performed. After tyrpsin or Asp-N protease

21  digestion, samples were analyzed by MALDI-TOF MS and a phosphopeptide containing

22  Y1357 was identified. In Figure 1C, the location of Y1357 relative to previously

23  identified serine/threonine phosphorylation sites is indicated (57). The major domains of

1    AIB1 necessary for interaction with other transcriptional components are also indicated

2    (3, 8, 13, 27, 31, 33, 49). The Y1357 site of SRC-3 is equivalent to: ACTR Y1345; AIB1

3    Y1353; RAC3 Y1350; TRAM-1 Y1357). The Y1357 site is located 67 amino acids (aa)

4    proximal to the C-terminus, juxtaposing a long polyglutamine tract (Figure 1C) and is

5    264 aa distal to the C-terminal end of the coactivator CBP/p300 (CID) binding site. The

6    Y1357 site and surrounding region has not been previously associated with any AIB1

7    functional domain. The Y1357 is also present in TIF-2/SRC-2 and in the mouse AIB1

8    homologue, p/CIP. Amino acids C-terminal to the Y1357, notably Q and S residues, are

9    also partially conserved in TIF-2/SRC-2 and p/CIP (Figure 1D).

10

11    **IGF-1 and EGF induce Y1357 phosphorylation in breast cancer cells.**

12    To confirm that the phospho-Y1357 site discovered by mass spectrometry analysis was

13    phosphorylated in vivo, a rabbit polyclonal antibody was generated against a peptide

14    containing the phospho-Y1357 residue and affinity purified. AIB1 was

15    immunoprecipitated from MCF-7 total lysate with this phospho-specific polyclonal

16    Y1357 antibody and the monoclonal AIB1 antibody was used for Western blot analysis

17    (Figure 2A). Phospho-Y1357 levels were significantly upregulated (2-5 fold) after either

18    IGF-1 or EGF treatments in all three cell lines examined (Figure 2A), indicating that the

19    phosphorylation of Y1357 was not limited to a single cell line or growth factor. A 10 to

20    30 minute treatment with either IGF-1 or EGF resulted in peak phospho-Y1357 levels,

21    without changing the total amounts of AIB1 protein (Figure 2A; lower "input" panels and

22    supplemental Figure S3). It was previously shown that estrogen (E2) treatments can

23    cause an increase in serine/threonine phosphorylation of AIB1 (57). We examined

1    whether estrogen induces phosphorylation of Y1357 in both ERα positive (MCF-7) and

2    ERα negative (MDA-MB-231) breast cancer cell lines. We found that phospho-Y1357

3    levels increased by ~2 fold after estrogen treatment without changing total AIB1 levels in

4    MCF-7 cells (Figure 2B; top panel). However, in MDA-MB-231 cells, we did not

5    observe estrogen induced phosphorylation at the Y1357 site (Figure 2B; lower panel).

6    Our results indicate that exposure to IGF-1, EGF or estrogen, in ERα positive cell lines,

7    can cause increased phosphorylation at Y1357 without changing total AIB1 protein

8    levels.

9    Since we observed a robust increase in phospho-Y1357 levels in breast cancer cells by

10   growth factor or estrogen treatment we asked whether phospho-Y1357 could be detected

11   in mammary tumors. To investigate this possibility, we examined by IHC the levels of

12   phospho-Y1357 in mammary tumors that develop in the MMTV-driven HER2/neu

13   transgenic mouse model. This model is strongly dependent on HER/ErbB receptor family

14   signaling for proliferation and metastasis (17). In these tumors, we observed a

15   significantly higher percentage of positive nuclei stained with the phospho-Y1357

16   antibody than in normal mammary epithelial cells, indicating that AIB1 (p/CIP) is highly

17   and selectively phosphorylated at residue Y1357 in these tumors (Figure 2C; "tumor" vs.

18   "wildtype"; see graph in lower right panel for quantitation).  The immunohistochemistry

19   was specific for Y1357 AIB1 since no nuclei were visibly stained in mammary glands

20   from SRC-3-/- (p/CIP-/-) mice with the phospho-Y1357 antibody. (Figure 2C; "SRC-3-/-

21   "left lower panel). Prior incubation of the phospho-Y1357 antibody with a peptide

22   containing the phosphorylated-Y1357 residue ("blocking peptide") also resulted in no

23   visible nuclei staining in both wildtype and tumor tissue sections (Figure 2C; middle

1     panels "wildtype+blocking peptide" vs. "tumor+blocking peptide"), further supporting

2     the specificity of the phospho-Y1357 antibody.

3

4     **Phosphorylation at Y1357 is necessary for AIB1's coactivator function.**

5     To help identify functions for phospho-Y1357, a phenylalanine mutant of Y1357 was

6     generated (Y1357F) and its effect on AIB1's ability to function as a transcriptional co-

7     activator was measured using several gene promoter reporters. Our analysis of the role of

8     the Y1357 mutation in these experiments were performed in both full length AIB1 and a

9     naturally occurring ~130 kDa MW AIB1-Δ3 isoform which differs from the full length

10    AIB1 by loss of the first 199 amino acids. We included the naturally occurring isoform

11    AIB1-Δ3 in addition to the full length AIB1 in our experiments to define the effect of

12    Y1357 because it has a significantly higher activity on a per mole basis than full length

13    AIB1 (42, 50). In addition, because of its lower molecular weight, the transfected AIB1-

14    Δ3 isoform can be detected in cell lines, such as COS-7 and HeLa cells, in which the

15    endogenous full length AIB1 is present at high levels (see supplemental Figure S2).

16    Compared to wildtype, the Y1357F mutant had ~50% coactivator activity on the estrogen

17    responsive (ERE) promoter reporter in the context of both full length AIB1 and AIB1-Δ3

18    (Figure 3A, left panel). The effect of the Y1357F mutant on AIB1's coactivation ability

19    was also assessed by measuring estrogen dependent induction of endogenous pS2 mRNA

20    levels in MCF-7 cells. Transient transfection of wild type AIB1 caused an increase in pS2

21    message, while no increase was observed with the Y1357 mutant in the presence of

22    estrogen (Figure 3A, right panel, real time qPCR). We also compared the effect of the

23    Y1357 mutant on another hormone responsive promoter, progesterone responsive

1  MMTV, and again observed that the Y1357F mutation impaired AIB1 and AIB1-Δ3's

2  coactivating function (Figure 3B).

3

4  The effect of the Y1357F mutant on steroid independent coactivation was tested with

5  multimerized NF-κB and AP-1 promoters. In the context of both AIB1 and AIB1-Δ3, the

6  Y1357F mutant caused a ~40% reduction in the activity of the NF-κB promoter

7  compared to the coactivating effect of wild type AIB1 and AIB1-Δ3. The reduction in

8  coactivator activity of the Y1357F mutant on an AP-1 promoter was also observed in the

9  context of full length AIB1 (Figure 3D). In contrast, we observed a ~3 fold increase in

10 activity of the AIB1-Δ3 Y1357F mutant on the AP-1 promoter (Figures 3C and D),

11 suggesting a role for the N-terminus of AIB1 in AP-1 mediated transcription. To

12 investigate the surprising effect of Y1357F mutation on AP-1 dependent expression

13 further, we analyzed its effect on a promoter fragment from the fibroblast growth factor

14 binding protein (FGF-BP) gene (19). The FGF-BP promoter is primarily AP-1 dependent

15 and is coactivated by AIB1 in the presence of EGF (42). Although the Y1357F mutant

16 activity was not significantly different than wild type AIB1-Δ3 in its ability to coactivate

17 this promoter (see supplemental Figure S3), there was a trend towards increased activity

18 even in the presence of a single AP-1 element in this promoter. The altered function of

19 the Y1357F mutant's ability to coactivate both hormone and growth factor responsive

20 promoters were not due to differences in exogenous AIB1 expressed protein levels (see

21 supplemental Figure S2). Overall these functional data indicate that phosphorylation at

22 Y1357 in AIB1 is important for both steroid dependent and independent transcriptional

1 control, although the impact of Y1357 phosphorylation is highly promoter-context

2 dependent.

3

4 **Phosphorylation of Y1357 alters AIB1 interaction with transcription cofactors.**

5 Since the phosphorylation status of Y1357 affected AIB1's coactivating ability on steroid

6 and NF-κB dependent promoters, we postulated that phosphorylation can affect

7 functional interactions between AIB1 and other proteins assembled in transcription

8 complexes formed in response to steroid hormones and growth factor signals. We first

9 examined interactions with the estrogen receptor, ERα. In immunoprecipitation assays,

10 we found ~50% less interaction between the Y1357F-FLAG mutant and ERα (Figure

11 4A1). However, when AIB1 and ERα were cotransfected together, we consistently

12 observed a slight reduction in total ERα levels occurred when cotransfected with Y1357F

13 mutant. To determine if the interaction between ERα and Y1357F was reduced due to an

14 alteration in their binding affinity and not due to a reduction in total ERα available for

15 interaction, we transfected the FLAG tagged AIB1 and ERα constructs separately into

16 293T cells and mixed the lysates in the presence or absence of estrogen and then

17 performed the FLAG IP followed by western blotting for ERα (Figure 4A2). Total

18 expression of levels of AIB1 and ERα were also evaluated in the input lysates. With

19 equal expression of ERα and AIB1, we observed a marked decrease in the affinity of

20 Y1357F mutant for ERα compared to wildtype AIB1 (Figure 4A2).

21

22 The interaction of AIB1 with CBP/p300, a histone acetyl-transferase, is also a critical

23 interaction for coactivation (8). HA-tagged p300 was co-transfected with FLAG-tagged

1     AIB1 or Y1357F mutant constructs and co-immunoprecipitations were performed with

2     the anti-FLAG antibody. Again, the Y1357F mutant interacted ~50% less than AIB1 in

3     this assay for binding to p300 (Figure 4B). We also noticed that Y1357 is close to a

4     CARM1 methylation and interaction site on AIB1 (Figure 1C). Unlike CBP/p300,

5     engagement of the CARM1 cofactor has been demonstrated to inhibit transcription

6     complex formation and to have a repressive effect on gene transcription (33). In contrast

7     to the interaction results with p300 and ERα, we observed slightly increased amounts of

8     CARM1 binding to the Y1357F mutant (Figure 4C) compared to non-mutated AIB1.

9     This result suggests that phosphorylation of this residue may play a minor role in

10     stabilizing the interaction of AIB1 with CARM1. Overall these data support the role of

11     Y1357 phosphorylation in controlling the interaction between AIB1 and cofactors, such

12     as ERα, p300 and CARM1, that ultimately alter its transcriptional activity.

13

14     Since mutation of Y1357 altered interactions with ERα and p300, we investigated

15     whether Y1357 phosphorylation caused discernible differences in AIB1's structure that

16     could explain changes in cofactor binding. Protein structure predictions were made with

17     the MODELLER 7v7 program and 300ps molecular dynamics simulations of the region

18     surrounding Y1357 and phospho-Y1357 were carried out using distant dependent

19     dielectric constants (Figure 4D). Upon phosphorylation both phospho-Y1357 and nearby

20     residues S1350, S1355, I1356, and E1361 (green backbone amino acids) move away

21     from one-another to avoid steric hindrance with the added, charged phosphate group,

22     illustrating possible structural and functional roles for both Y1357 and phospho-Y1357.

23     Therefore, phosphorylation at Y1357 could cause local structural alterations that increase

1     the stability of AIB1's interactions with transcription machinery components, such as

2     p300 and ERα, while dephosphorylation could maintain the stability of CARM1 binding,

3     at the expense of p300 and ERα binding.

4

5     **AIB1 Y1357 is phosphorylated by the Abl kinase pathway**

6     Since we determined that phosphorylation at Y1357 had a functional role for AIB1's

7     ability to coactivate by promoting the formation of transcription cofactor complexes, we

8     wanted to determine the tyrosine kinase that was responsible for Y1357 phosphorylation.

9     To narrow down the possible tyrosine kinases that could phosphorylate AIB1, the amino

10    acid sequences around Y1357 were analyzed using Scansite 2.0 software program to

11    determine if the sequences formed a consensus substrate for a particular tyrosine kinase

12    (36). The Scansite program predicted that the Y1357 and surrounding residues in AIB1

13    was a possible Abl tyrosine kinase substrate based on the presence of Isoleucine at

14    position –1 to Y residue which was also found in other substrates of Abl kinase e.g. Dok

15    (60), and Cas (44) (Figure 5A). A general consensus for Abl kinase phosphorylation

16    substrate has been derived from six known substrates (4, 10, 12, 44, 60, 63) (Figure 5A).

17    Interestingly, the Isoleucine at position -1 was a given a higher selectivity value

18    compared with the Proline +3 in a study that originally characterized Abl's substrate

19    sequence specificity (47). However, it appears from the comparison in Figure 5A that the

20    Proline +3 is a common feature of many known Abl substrates. To determine if AIB1

21    was indeed phosphorylated by Abl kinase we first performed an in vitro kinase assay to

22    determine if a GST fragment containing the Y1357 residue could be phosphorylated by

23    recombinant Abl kinase. We found that a GST-AIB1 fragment 1017 to 1420 aa was

1    readily phosphorylated at Y1357 by exogenous Abl kinase, as detected by the phospho-

2    Y1357 antibody (Figure 5B). To confirm that Abl kinase could phosphorylate AIB1 in

3    whole cells, we overexpressed Abl kinase using an Abl-AU5 tagged construct and co-

4    transfected it with an AIB1-FLAG construct into 293T cells. We immunoprecipitated

5    AIB1 with either a FLAG or phospho-Y1357 antibody and detected phosphorylated

6    AIB1 by western blot. Since CrkL is an Abl/Bcr-Abl substrate (11), phospho-CrkL

7    (Y207) levels were measured (Figure 5C, input panels) to ensure that the transfected Abl

8    kinase was functional. Consistent with the in vitro kinase assay, we detected a high

9    amount of Y1357 phosphorylation only in the presence of transfected active Abl kinase

10   (Figure 5C, IP:FLAG panels).

11   Abl has the ability to phosphorylate and bind directly to its substrate targets, such as c-

12   Jun (4)  and Cas (44). We therefore determined if Abl has the ability to complex with

13   AIB1 and if this binding was affected by the phospho-Y1357 residue. We co-transfected

14   Abl with either AIB1 or the AIB1 Y1357F mutant into 293T cells and examined their

15   interaction with Abl kinase by co-immunoprecipitation and western blot analysis. Abl

16   interacted strongly with AIB1 and ~50% of this binding was lost between Abl and the

17   Y1357F mutant (Figure 5D, IP:FLAG, WB:AU5, lane 3). This result indicated that

18   phosphorylation of the Y1357 residue increased the affinity for Abl kinase, but was not

19   absolutely required for the AIB1 interaction with Abl kinase. Like other non-receptor

20   tyrosine kinases, Abl mainly exists intracellularly in an inactive form and becomes

21   activated by either external signals such as growth factor stimulation, cell adhesion or as

22   a response to DNA damage (as reviewed in (39)). Conversely, the Abl kinase inhibitor,

23   imatinib (Gleevec[®]; STI-571), binds to the ATP binding pocket when Abl is in its

1    inactive conformation (45). To determine if the activation of Abl kinase was necessary

2    for the interaction with AIB1, 293T cells were pretreated with, imatinib 1 hr prior to

3    harvesting the cells for immunoprecipitation analysis. Inhibition of Abl kinase activity

4    eliminated phosphorylation at Y1357 and completely prevented the interaction between

5    Abl and AIB1 (Figure 5D, IP:FLAG, WB:AU5, lane 4). This result strongly suggests that

6    AIB1 can only interact with the active form of Abl kinase. The inhibition of Abl kinase

7    activity by imatinib was confirmed by measuring phospho-CrkL levels (Figure 5D, input,

8    lane 4).  Since we observed that estrogen could increase the phosphorylation of the

9    Y1357 site (Figure 2B), and that conversely mutation of the Y1357 site diminished ERα

10   interaction with AIB1 interaction (Figure 4A), we were also interested to determine how

11   increasing Abl kinase activity would affect the ERα/AIB1 complex formation. To

12   accomplish this we transfected 293T cells with a combination of ERα, Abl-AU5 and

13   AIB1-FLAG expression constructs and determined by immunoprecipitation and western

14   blot analysis the amount of ERα/AIB1 complex formation in the presence or absence of

15   added estrogen. As expected, immunoprecipitation of ERα brings down AIB1 and this

16   interaction is increased in the presence of 10 nM estrogen (Figure 5E, lane 5).  Some

17   interaction with ERα and AIB1 was observed in the absence of estrogen. Due to the high

18   expression of transfected ERα, residual estrogens in the charcoal stripped serum media

19   was enough to cause some ERα/AIB1 complex formation (Figure 5E, lanes 2 and 3).

20   Interestingly, when Abl kinase is active a significant increase in the amount of complex

21   between ERα and AIB1 occurs (Figure 5E, lane 6). Consistent with the idea that Abl

22   phosphorylates AIB1, we also observed a significant upward mobility shift in the

23   immunoprecipitated AIB1 in the lanes where Abl kinase is overexpressed (Figure 5E,

lanes 3 and 6). These data suggest that phosphorylation of AIB1 by Abl kinase facilitates the interaction with ERα and this is considerably enhanced in the presence of estrogen. To confirm that Abl kinase phosphorylates AIB1 in a breast cancer cell line, we used an siRNA directed against endogenous Abl kinase to determine if reducing Abl kinase levels/activity resulted in a corresponding decrease in phospho-Y1357 levels. As shown in Figure 2A, EGF treatment in MDA-231 cells resulted in an increase in phospho-Y1357 levels. When Abl kinase activity was reduced in MDA-231 cells with siRNA transfection, phospho-Y1357 levels were reduced dramatically (Figure 5F). Total levels of Abl were difficult to detect in MDA-231, therefore phospho-CrkL activation was used as a surrogate marker for Abl siRNA knockdown. We observed a 20-40% decrease in phospho-CrkL levels when transfected with the Abl siRNA (Figure 5F). These data clearly indicate that the Y1357 site on AIB1 is a substrate for Abl kinase in breast cancer cells.

**Abl activity and phospho-Y1357 site contribute to AIB1's function as a criticial coactivator and role in tumorigenesis.**

To assess its effect on AIB1 coactivator activity in MCF-7 cells, we inhibited endogenous Abl in MCF-7 cells with imatinib. MCF-7 cells carry the AIB1 gene amplification and therefore express very high amounts of AIB1 protein. Imatinib inhibited both basal and exogenous AIB1 coactivation of a MMTV promoter reporter in the presence of R5020 (Figure 6A). AIB1 is rate-limiting for estrogen induced growth of MCF-7 cells (28). Imatinib or Abl siRNA treatment significantly reduced MCF-7 cell growth after 4 days of estrogen treatment (Figure 6B). These findings demonstrate that Abl activity is necessary

1  for AIB1's coactivation of hormone dependent gene promoters and, ultimately, necessary

2  for hormone dependent growth of breast cancer cells. To directly assess the Y1357 site's

3  contribution to AIB1 dependent tumorigenesis, focus formation assays were performed

4  with transiently transfected H-ras V12 and the Y1357F mutant constructs in AIB1/SRC-3

5  -/-  mouse embryonic fibroblasts (MEFs). AIB1 has been shown to reduce the incidence

6  and latency of breast tumors in the MMTV v-Ha-ras mammary tumorigenesis mouse

7  model (24). Wildtype AIB1 alone or the Y1357F mutant did not induce focus formation

8  (data not shown) while H-ras V12 alone did result in the formation of a limited number of

9  foci. Wildtype AIB1 plus H-ras V12 produced an increased number of foci, while the

10  Y1357F mutant plus H-ras V12 produced fewer foci (Figure 6C; chart). These data

11  demonstrate that Y1357 site directly contributes to AIB1's role in an oncogene dependent

12  transformation assay. We propose a molecular model (Figure 6D) in which activated Abl

13  binds to and phosphorylates AIB1 at Y1357. Y1357 phosphorylated AIB1 leads to a

14  conformational alteration that stabilizes AIB1's interaction with cofactors such as ERα

15  and p300, while simultaneously resulting in a less stable interaction with CARM1.

16  Phosphorylation at Y1357 is required for AIB1's ability to mediate steroid receptor

17  dependent gene transcription as well as contribute to its role in breast cancer

18  tumorigenesis.

19

20  **DISCUSSION**

21  This is the first study, to our knowledge, that describes the tyrosine phosphorylation of a

22  steroid receptor coactivator. Although AIB1 tyrosine phosphorylation is initiated by

23  membrane tyrosine kinases it appears to be eventually mediated by Abl, a non-receptor

1    tyrosine kinase. Our results are consistent with a model outlined in Figure 6D whereby

2    Abl kinase is activated by an extracellular signal and in its activated form creates a

3    complex with AIB1. AIB1 is then rapidly phosphorylated by Abl at tyrosine Y1357

4    thereby changing its local conformation and increasing its affinity for p300 and steroid

5    receptors and decreasing its affinity for the repressor CARM1. At promoters that harbor

6    estrogen, progesterone or NF$\kappa$B response elements this leads to an overall increase in

7    transcription. At other promoter elements, such as AP-1 sites, the tyrosine

8    phosphorylation of AIB1 seems to be less important in formation of the transcription

9    complex and may normally even repress transcription. This suggests that other AIB1

10   cofactor interactions may play a rate-limiting role in this promoter context. Interestingly

11   it has been shown that AP-1 mediated transcription is impacted by serine and threonine

12   phosphorylation of AIB1  (57). Furthermore, it has been postulated that phosphorylation

13   at a particular residue of AIB1 may be a driving event enabling subsequent post-

14   translation modifications (55). It would of interest to determine if Y1357 is a primary

15   permissive phosphorylation or is a secondary occurrence after other post translational

16   modifications including as yet uncharacterized additional tyrosine, serine and threonine

17   phosphorylation sites in AIB1. Tyrosine phosphorylation is usually a consequence of

18   rapid activation of growth factor receptor tyrosine kinases and cytoplasmic protein

19   tyrosine kinases upon ligand stimulation. Therefore, it may be more likely that tyrosine

20   phosphorylation of AIB1 is an early rate limiting modification which influences

21   phosphorylation or post-translational modifications at other sites.

22   The phosphorylation of AIB1 by Abl kinase was a somewhat surprising result especially

23   as the Abl kinase consensus surrounding the Y1357 residue is not highly conserved.  The

1     role of Abl kinase in oncogenesis is complex. The oncogenic forms for Abl, v-Abl and

2     Bcr-Abl, have been extensively studied and well described; however, the normal cellular

3     functions of Abl are still being characterized (39, 52). Unlike Src tyrosine kinase, Abl

4     has been found to have both a cytoplasmic and nuclear function and it has profoundly

5     different functions depending on is subcellular localization. Cytoplasmic Abl is

6     associated with cell growth, motility, migration, and adhesion; while nuclear Abl is

7     associated with apoptosis (15, 52). Similar to Abl kinases, AIB1 is also both a

8     cytoplasmic and nuclear protein, albeit the full length protein appears to be

9     predominantly nuclear (29, 41) The mechanisms which alter the localization of AIB1

10     have been a topic of intense focus as it may be important in regulating post-translational

11     modifications as well as protein stability of AIB1. (2, 26, 62). It would be of interest to

12     determine if the Abl-AIB1 interaction and phosphorylation occurs in a specific

13     subcellular compartment, what other modifications precede or follow Y1357

14     phosphorylation, as well as the resulting functional consequences.

15     Our results strongly suggest that phosphorylation of and interaction with AIB1 by Abl

16     kinase plays a role in either Abl or AIB1 mediated oncogenesis. As stated above, Abl can

17     have different roles in oncogenesis depending on its subcellular localization and also the

18     level of its activated expression. Similarly AIB1 can be oncogenic when overexpressed in

19     mammary epithelium and other epithelial tissue (50, 51, 61). Conversely AIB1/SRC-3 -/-

20     transgenic mice develop lymphomas as they age (9) suggesting that in this context AIB1

21     may normally suppress oncogenesis. It would be of interest to determine if different

22     functional interactions between Abl and AIB1 in the hematopoietic system compared

23     with epithelial cells alters the role of AIB1 in oncogenesis. It may be possible that an

1  epithelial tissue growth factor and steroid receptor pathways activate Abl and thus AIB1.

2  However, in the hematopoietic system a different paradigm may operate between Abl and

3  AIB1 possibly in a different subcellular compartment. These are intriguing questions for

4  further study.

5  Abl is activated by PDGF and EGF (40), but whether IGF-1 or insulin are possible

6  activators of Abl kinase seem to be somewhat cell line dependent and are still not fully

7  understood (14, 46, 48). Regardless of the extracellular activator of Abl kinase, we

8  postulate that other intracellular Abl activated proteins (Figure 6D) will be a necessary

9  part of the Abl-AIB1 complex. Abl usually exists in an inhibited state in which either Abl

10  keeps its kinase domain and SH2/SH3 domain tightly bound to itself (18, 35) or by

11  binding to inhibitory proteins, such as ABI-1 (39). Activation of Abl kinase, perhaps due

12  to phosphorylation (6, 34), results in exposure of the N-terminal myristoyal group and

13  exposure of the SH2/SH3 domains to bind to phospho-tyrosine proteins. It has been

14  postulated that Abl substrates are initially phosphorylated by basal kinase activity of Abl,

15  which initiate a positive feedback loop by activating SH2 domain dependent activation of

16  Abl and finally results in the recruitment of its substrate (18). Discovering the

17  components of the AIB1-Abl kinase complex, especially a SH2/SH3 domain containing

18  protein that also binds to AIB1 may add further levels of complexity to the regulation of

19  AIB1 function.

20  A possible clinical application of this study is the utilization of the phospho-specific

21  antibody to detect phosphorylated AIB1 at Y1357 as a marker for activated Abl kinase in

22  tumors and possible responsiveness to Abl kinase inhibitors such as imatinib. At the

23  writing of this article, seven clinical trials were ongoing to study the beneficial effects of

1    using imatinib in conjunction with other therapies to treat metastatic breast cancer. One

2    of the inclusion criteria of these trials is the presence of molecular markers, c-kit and

3    PDGFRb. Autocrine PDGF/ PDGFR signaling has been shown to promote metastasis in

4    MMTV-Neu transgenic mice and imatinib treatment was shown to reduce metastasis

5    (21). This finding is interesting since we also observed an increase in activated phospho-

6    Y1357 AIB1 in HER2/neu tumors (Figure 2C), thus suggesting that AIB1 may be

7    downstream of PDGFR signaling. It will be interesting to determine in patient samples

8    the levels of tyrosine phosphorylated AIB1 and whether this is predictive of outcome in

9    therapies directed at reducing growth factor and/or Abl kinase signaling. Since Abl

10   kinase promotes complex formation between ERα and AIB1, as well as reducing NFκB

11   mediated transcription, imatinib may have an inhibitory effect on mammary tumor

12   growth in both steroid dependent and independent settings in breast cancer. Finally due to

13   the successful use of imatinib in the treatment of multiple human leukemias and the

14   emergence of imatinib resistance in patients, a large number of drugs that target Abl,

15   PDGFRb and Src are in the pipeline for drug development and testing. These inhibitors

16   may also be applicable in the treatment of breast cancer especially those that have high

17   levels of phospho-Y1357 AIB1.

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19

20

21

**FIGURE LEGENDS**

**FIG 1. Growth factor induced tyrosine phosphorylation of AIB1.**

(A) IGF-1 induced tyrosine phosphorylation of AIB1 in MCF-7 breast cancer cells. Cells

were serum starved for 24 hrs and treated with 50 ng/ml IGF-1 for 10 min. Whole cell

lysates were harvested and used for immunoprecipitation (IP) and western blot (WB)

analysis with antibodies as indicated. (B) EGF induced tyrosine phosphorylation of AIB1

in MDA-MB-231 breast cancer cells. Cells were stimulated for 10 min with 50 ng/ml of

EGF and then processed and analyzed as in (A). (C) A schematic of AIB1/SRC-3 protein

showing conserved and functional domains, serine and threonine phosphorylation sites

and the region containing multiple methylation sites. Phosphorylation at the Y1357

residue was discovered utilizing mass-spectrometry. SRC-3 amino acid numbering was

used for consistency. (D) Comparisons of amino acids surrounding Y1357 in AIB1 with

other p160 family members: SRC-1, TIF2/SRC-2, and p/CIP, the mouse homologue of

human AIB1. Conserved amino acids are highlighted.

**FIG 2. In vitro and in vivo detection of phospho-Y1357 AIB1.**

(A) Phospho-Y1357 phosphorylation is observed in breast and lung cancer cell lines

following growth factor stimulation using the phospho-Y1357 antibody. Cells were

treated with 50 ng/ml of IGF or EGF for 10 min. Whole cell lysates were harvested and

used for IP/WB analysis with antibodies as indicated. (B) Estrogen (E2) induced

phospho-Y1357 levels in MCF-7 (ER+; upper panel) cells but not in MDA-231 (ER-;

lower panel) cells. Hormone-stripped cells were treated for 30 min with either EtOH or

E2 (10 nM) before whole cell lysates were harvested for IP/WB analysis. (C) Increased

1    phospho-Y1357 levels were observed in HER2/neu tumor tissue. Typical IHC staining

2    patterns for phospho-Y1357 expression in paraffin embedded mammary gland sections

3    from female mice at 11 months (HER2/neu, "tumor", n=3) and from normal mammary

4    gland #4 from mice at 6 months (SRC-3 wt/wt, "wildtype", n=3 or "SRC-3-/-", n=2).

5    The phospho-Y1357 blocking peptide and phospho-Y1357 antibody were incubated

6    together on each tissue section for 30 min. 80-100 epithelial cells were counted per field.

7    10 fields counted per genotype. **p<0.0022; unpaired t test. Error bars indicate mean ±

8    S.D.

9

10   **FIG 3. Functional role for phospho-Y1357 in steroid-dependent and -independent**

11   **transcription** (A) left panel.Y1357F mutant coactivator effect on estrogen stimulated

12   transcription.  AIB1 and AIB1-Δ3 constructs were cotransfected with ERα and ERE

13   reporter construct into hormone stripped COS-7 cells. Cells were treated with EtOH or

14   E2 10 nM for 24 hrs and analyzed for reporter activity. *p<0.03, #p<0.0012; unpaired t

15   test. (A) right panel. ERα (0.5 ug) and AIB1 (3 ug) constructs were cotransfected into

16   MCF-7 cells for 24 hrs and treated with E2 for 3 hrs. Total RNA was harvested and pS2

17   and beta-actin mRNA levels were measured using reverse transcription quantitative real

18   time PCR. *p<0.01, #p<0.001; unpaired t test. (B) Y1357F mutant coactivator activity

19   was measured on progesterone dependent promoter. PR-B expression plasmids were co-

20   transfected with the MMTV reporter plasmids into hormone-stripped CHO cells. Cells

21   were treated with either EtOH or10 nM R5020 for 24 hrs and then analyzed for reporter

22   activity. n=3; *p<0.0012 and #p<0.0007; two-way ANOVA. (C and D) Y1357F mutant's

23   coactivator effects on steroid-independent promoters. HeLa cells were co-transfected

with AIB1 expression constructs as indicated and either (C) a multimerized NF-κB

reporter construct (Stratagene Co.) or (D) a multimerized AP-1 reporter construct

(Stratagene Co.). 25 ng of c-fos and c-jun expression vectors were also co-transfected

with the AP-1 reporter. 24 hrs after transfection, extracts were prepared for reporter

assays. Results are expressed as fold activation of empty vector transfected cells. n=3;

*#p<0.01; unpaired t test. Results are expressed as fold activation of empty vector

transfected cells; error bars indicate mean ± S.D.


**FIG 4. Phosphorylation of Y1357 modulates transcription cofactor interactions.**

Interaction of the AIB1 Y1357F mutant with transcription cofactors: (A1 and A2) ERα,

(B) p300-HA, and (C) CARM1-HA. ERα, p300-HA and CARM1-HA expression

plasmids were separately co-transfected with AIB1-Δ3-FLAG ("AIB1 (FLAG)")

constructs in 293T cells and whole cell lysates were prepared 24 hrs later for IP/WB

analysis. The ratio of the amount of non-mutated AIB1-FLAG immunoprecipitated with

the target protein (ERα, p300 or CARM1) was standardized to 1 and compared with the

ratio of Y1357F mutant FLAG immunoprecipitated with the target protein. In panel (A1)

293T cells were treated with EtOH or 10 nM E2 for 1 hr before whole cell lysates were

prepared for analysis. In panel (A2) ERα and AIB1 constructs were transfected

separately into 293T cells and lysates made. ERα and AIB1 containing lysates were

mixed and either treated with EtOH or E2 100 nM before immunoprecipitation was

performed. (D) Simulated effect of phosphorylated Y1357 on the local structure of AIB1.

White backbone amino acids represent the *unphosphorylated* state; green backbone

amino acids represent the *phosphorylated* state.

1

**FIG 5. Abl kinase directly phosphorylates phospho-Y1357 and binds to AIB1.**

(A) AIB1 Y1357 contains a partial Abl kinase recognition site. Amino acids immediately surrounding the AIB1 Y1357 residue were compared to an Abl kinase consensus sequence peptide and known Abl kinase substrates. Amino acids that are identically positioned are highlighted. (B) Abl kinase phosphorylates an AIB1 GST fragment in vitro. An in vitro kinase assay was performed with purified GST-AIB1 (1017-1420 aa) protein and recombinant Abl kinase. (C) Expression of constitutively active Abl phosphorylates AIB1 at Y1357. Abl-AU5 was co-transfected with AIB1-Δ3-FLAG constructs (AIB1 or Y1357F) in 293T cells. P-CrkL (Y207) levels were detected to determine Abl activation. (D) Interaction between Abl and AIB1 is partially mediated by Y1357 and is fully dependent on Abl kinase activity. Abl-AU5 and AIB1-Δ3-FLAG (AIB1 or Y1357F) constructs were used as in panel (C). Transfected 293T cells were pretreated for 4 hrs with either DMSO or 10 μM imatinib prior to collection of lysates and IP. (E) Abl forms a complex with ERα and AIB1 in the presence of estrogen. 293T cells were transfected with Abl-AU5, ERα, and AIB1-Δ3-FLAG for 24 hrs and treated with either EtOH or 10 nM E2 before whole cell lysates were harvested. Lysates were immunoprecipitated with ERα followed by western blot analysis for FLAG or ERα. (F) Reduction of Abl results in a decrease in endogenous AIB1 Y1357 phosphorylation in MDA-231 cells. MDA-231 cells were transfected with Abl (exon 11) siRNA for 48 hrs, serum starved and treated 10 min with vehicle or EGF. P-CrkL levels were used to assess reduction in Abl activity.

23

1 **FIG 6. Abl activity is necessary for AIB1's role in hormone induced promoter**

2 **coactivation and proliferation of breast cancer cells.** (A) Inhibition of Abl kinase

3 activity by imatinib reduces AIB1's ability to coactivate progesterone dependent gene

4 promoter activity. MCF-7 cells were transfected with MMTV reporter, PR-B, AIB1

5 vectors for 24 hrs, pretreated 1 hr with 10 μM imatinib and then treated with 10 nM

6 R5020 with 10 μM imatinib for an additional 24 hrs prior reporter analysis. n=3;

7 **p<0.002; two-way ANOVA. Results are expressed as fold activation of empty vector

8 transfected cells. (B) Inhibition of Abl kinase significantly reduces E2 induced cell

9 growth of MCF-7 breast cancer cells. For imatinib growth assays, hormone-stripped

10 MCF-7 cells were pretreated 1 hr with 10 μM imatinib and then treated with EtOH or 10

11 nM E2 with imatinib for 4 days. For Abl siRNA growth assays, hormone-stripped MCF-7

12 cells were transfected with scrambled (con.), abl.3 or abl.11 siRNAs (specific for exon 3

13 or 11) for 24 hrs. Cells were treated with EtOH or 10 nM E2 for 4 days. Each experiment

14 was performed in triplicate. *p<0.001, **p<0.0002; two-way ANOVA. (A and B) Error

15 bars indicate mean ± S.D. (C) Y1357F mutant demonstrates reduced H-rasV12 dependent

16 focus formation in AIB1/SRC-3 -/- MEFs. AIB1/SRC-3 -/- MEFs was transfected with

17 H-ras V12 and empty vector, AIB1-Δ3 (WT) or AIB1-Δ3 Y1357F (Y1357F) constructs.

18 After 3 weeks, focus formation was assessed after staining with crystal violet. 3

19 independent experiments were performed. (D) A proposed model for the role of Abl

20 tyrosine phosphorylation of AIB1 in steroid receptor signaling. Activated Abl binds to

21 and phosphorylates AIB1 at Y1357. Phospho-Y1357 AIB1 stabilizes its interaction with

22 cofactors such as ERα and p300, while simultaneously resulting in a less stable

23 interaction with CARM1.

# Fig.1, Oh et al.

## A
**MCF-7**

IGF-1:    -    +

IP: AIB1 [ \* WB: PY ]
[ WB: AIB1 ]

## B
**MDA-MB 231**

IP:  AIB1  IgG
EGF:  -    +

WB: PY

WB: AIB1

## C
**AIB1**

**Interaction Domains**

Methylation (1151-1210)

PAS   EID   RID                    CID              Q-rich

bHLH  116-171  334-444  621-625  684-688  738-742          1020  1093   1169   poly Q  1313

16  88  267-302

1                                                                              1424

A  B

^           ^^        ^^^            ^
T24        S505      S857          Y1357
           S543      S860
                     S867

**Phosphorylation sites (^)**
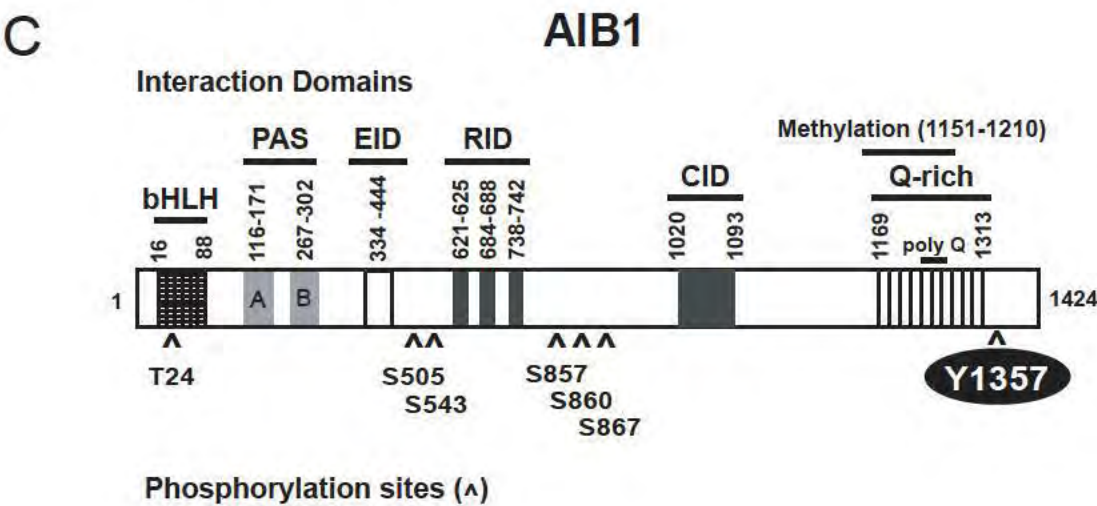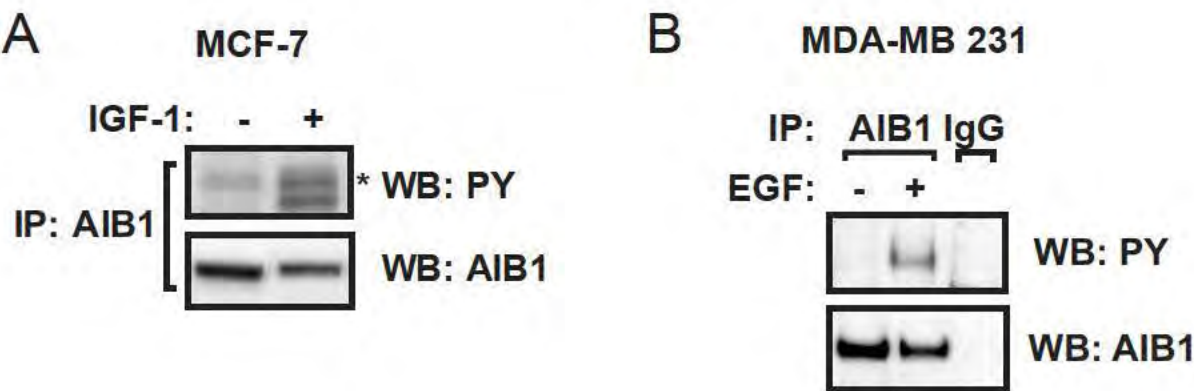
## D

```
AIB1  :1337 SSRMGPSQNPMMQHPQAASIY^QS-SEMKGWPSGNLARNSSFSQQ
SRC-1 :1337 QMQMSSLQMPGMNTVCPEQIN-DPALRHTGLYCNQLSSTDLLKTE
TIF2  :1327 SPRMAHTQSPMMQQSQANPAY^QAPSDINGWAQGNMGGNSMFSQQ
p/CIP :1311 SSRMGPSQNAMVQHPQPTPMY^QP-SDMKGWPSGNLARNGSFPQQ
```

Fig. 2, Oh et al.
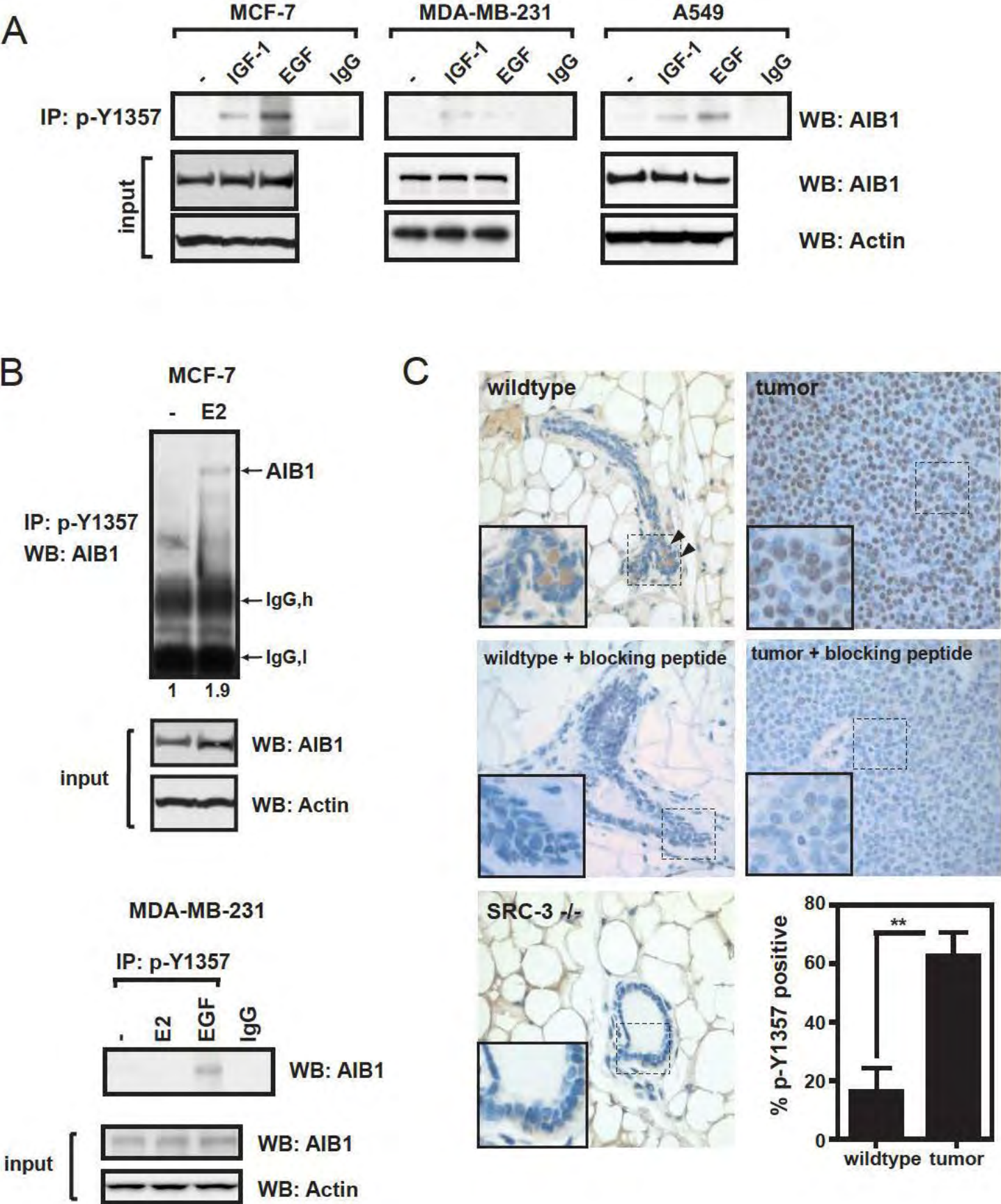
Fig. 3, Oh et al.

# Fig.4, Oh et al.

## A1

AIB1 (FLAG)　　Y1357F (FLAG)

E2 10 nM :  -  +   -  +

ERα :  +  +   +  +

WB: ERα

1    0.5

IP: FLAG

WB: FLAG

input     WB: ERα

## B

AIB1 (FLAG) :  -  +  -

Y1357F (FLAG) :  -  -  +

p300 (HA) :  +  +  +

WB: HA

IP:FLAG     1  0.5

WB: FLAG

input     WB: HA

## A2

transfected constructs :  -  WT  Y1357F

FLAG

actin

-  ERα

ERα

actin

total protein expression in lysates

↓

mixed ER and FLAG lysates

↓

| + | + |   |   |   |   | : - |
|   |   | + | + |   |   | : WT |
|   |   |   |   | + | + | : Y1357F |
| + | + | + | + | + | + | : ERα |
| - | + | - | + | - | + | : E2 |

lysate

WB: ERα

IP: FLAG

WB: FLAG

## C

AIB1 (FLAG) :  -  +  -

Y1357F (FLAG) :  -  -  +

CARM1 (HA) :  +  +  +

WB: HA

IP:FLAG     1  1.2

WB: FLAG

input     WB: HA

## D



phospho-Y1357

Y1357

S1350

S1355

I1356

E1361

K1363

Deviation 2.33Å at 300ps

# Fig. 5, Oh et al.

## A

| | | | position | | | | |
|---|---|---|---|---|---|---|---|
| | -3 | -2 | -1 | 0 | +1 | +2 | +3 |
| AIB1/SRC-3: | A | S | I | Y | Q | S | S |
| c-Abl site : | X | V | I/V/L | Y | A | A | P |

known substrates:

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| c-Jun: | P | P | V | Y | A | N | L |
| Crk : | P | G | P | Y | A | Q | P |
| p73 : | H | S | P | Y | A | Q | P |
| Dok : | D | P | I | Y | D | E | P |
| Cbl : | D | D | G | Y | E | V | P |
| Cas : | Q | D | I | Y | D | V | P |

## B



WB: p-Y1357

Ponceau S

GST — 1017 ... 1420

## C

| pCMV : | + | - | + | - |
|---|---|---|---|---|
| AIB1 (FLAG) : | - | + | - | + |
| Abl (AU5) : | - | - | + | + |

IP: FLAG — WB: p-Y1357 / WB: FLAG

IP: p-Y1357 — WB: FLAG

input — WB: AU5 / WB: p-CrkL / WB: Actin

## D

+Abl (AU5)

| pCMV : | + | - | - | - |
|---|---|---|---|---|
| AIB1 (FLAG) : | - | + | - | + |
| Y1357F (FLAG) : | - | - | + | - |
| imatinib 10 uM : | - | - | - | + |

IP: FLAG — WB: p-Y1357 / WB: AU5 / WB: FLAG

IP: AU5 — WB: FLAG / WB: AU5

input — WB: p-CrkL / WB: Actin

lane: 1 2 3 4

## E

E2 (10 nM)

| pCMV : | + | - | - | + | - | - |
|---|---|---|---|---|---|---|
| AIB1 (FLAG) : | - | + | + | - | + | + |
| Abl (AU5) : | - | - | + | - | - | + |
| ERα : | + | + | + | + | + | + |

IP: ERα — WB: FLAG / WB: ERα

input — WB: FLAG / WB: AU5 / WB: p-CrkL / WB: Actin

lane: 1 2 3 4 5 6

## F

| siRNA : | control | | abl | | IgG |
|---|---|---|---|---|---|
| :EGF 50 ng/ml | - | + | - | + | - |

IP: p-Y1357
WB: AIB1 — 150 kDa — non-specific band

total lysate — WB: p-CrkL
1    1.1    0.6    0.8
WB: actin

# Fig. 6, Oh et al.

## A

**MMTV**



| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| - | + | - | + | - | + | - | + | : AIB1 |
| - | - | - | - | + | + | + | + | : R5020 |
| - | - | + | + | - | - | + | + | : imatinib |

## B

**MCF-7 cell growth**



| 1 | 0.49 | 0.48 |

## C

**# of foci formed in SRC3-/- MEFs**

| | Expt. No. | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| empty vector | 0 | 0 | 1 |
| H-ras V12 | 3 | 11 | 3 |
| H-ras V12 + WT | 14 | 31 | 16 |
| H-ras V12 + Y1357F | 8 | 14 | 2 |



empty        H-ras V12

WT +         Y1357F +
H-ras V12    H-ras V12

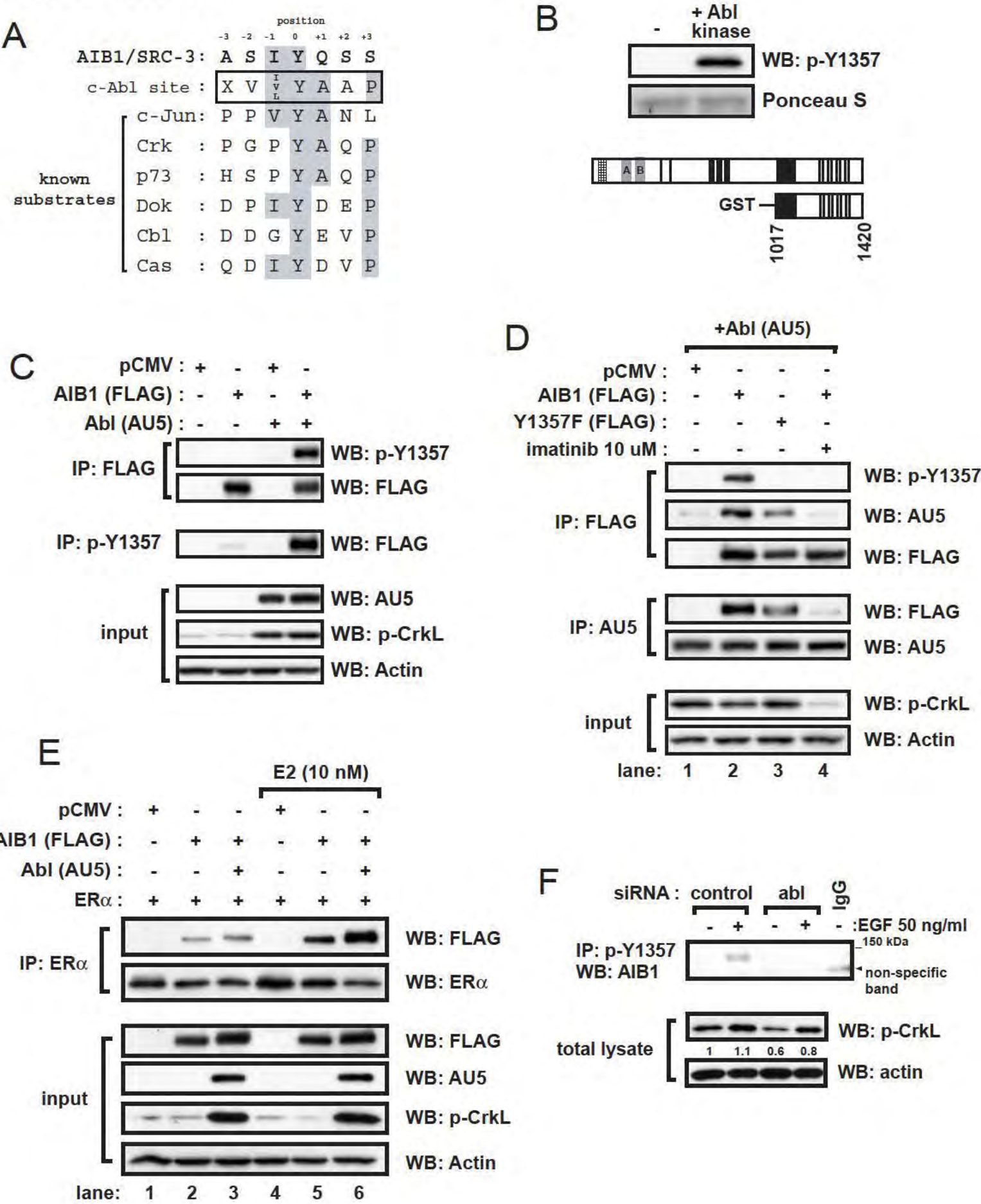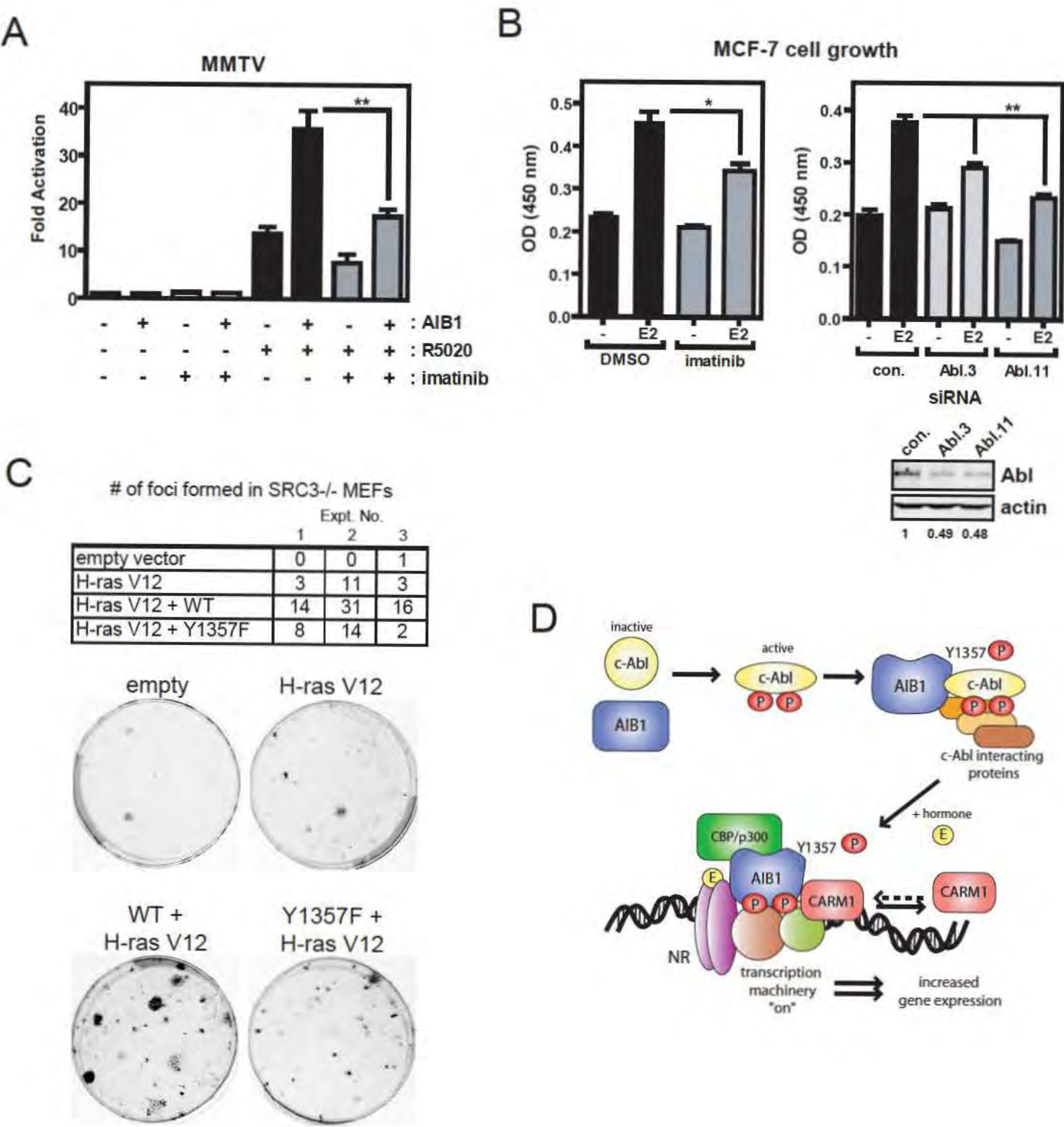## D

Buthionine Sulfoximine Sensitizes Hormone-Resistant Human Breast Cancer Cells to Estrogen-Induced Apoptosis

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**Running Title**: glutathione depletion and estrogen-induced apoptosis in breast cancer

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**Abstract**

**Introduction**

Estrogen deprivation using aromatase inhibitors is one of the standard treatments for postmenopausal women with estrogen receptor (ER)-positive breast cancer, however, one of the consequences of prolong estrogen suppression is acquired drug resistance. Our laboratory is interested in studying antihormone resistance and has previously reported the development of an estrogen deprived human breast cancer cell line, MCF-7:5C, which undergoes apoptosis in the presence of estradiol. In contrast, we have another estrogen deprived cell line, MCF-7:2A, which appears to have elevated levels of glutathione (GSH) and is resistant to estradiol-induced apoptosis. In the present study, we evaluated whether buthionine sulfoximine (BSO), a potent inhibitor of glutathione (GSH) synthesis, is capable of sensitizing antihormone resistant MCF-7:2Ac ellst oe stradiol-induceda poptosis.

**Methods**

Estrogen deprived MCF-7:2A cells were treated with 1 nM $E_2$, 100 μM BSO, or 1 nM $E_2$ +100 μM BSO combination *in vitro*, and the effects of these agents on cell growth and apoptosis were evaluated by DNA quantitation assay and annexin V and TUNEL staining. The *in vitro* results oft he MCF-7:2A celll inew eref urtherc onfirmed *inv ivo* ina m ousex enograftm odel.

**Results**

Exposure of MCF-7:2A cells to 1 n*M* $E_2$ plus 100 μ*M* BSO combination for 48-96 hours produced a 7-fold increase in apoptosis whereas the individual treatments had no significant effect on growth. Induction of apoptosis by the combination treatment of $E_2$ plus BSO was evidenced by changes in Bcl-2 and Bax expression. The combination treatment also markedly increased phosphorylated JNK levels in MCF-7:2A cells and blockade of the JNK pathway

attenuated the apoptotic effect of $E_2$ plus BSO. Our *in vitro* findings corroborated *in vivo* data from a mouse xenograft model in which daily administration of BSO either as a single agent or inc ombinationw ithE $_2$s ignificantly reducedt umorgr owthof M CF-7:2Ac ells.

**Conclusion**

Our data indicates that GSH participates in retarding apoptosis in antihormone-resistant human breast cancer cells and that depletion of this molecule by BSO may be critical in predisposing resistant cells to $E_2$-induced apoptotic cell death. We suggest that these data may form the basis of improving therapeutic strategies for the treatment of antihormone resistant ER-positive breast cancer.

**Introduction**

Currently, estrogen deprivation using aromatase inhibitors is one of the standard treatments for postmenopausal women with ER-positive breast cancer [1]. Unfortunately, a majorc linicalpr oblemw itht heus eof pr olonged estrogend eprivationi st hede velopmentof drug resistance (i.e. hormone independent growth) [2, 3]. Our laboratory as well as other investigators have a major effort in studying antihormone resistance in breast cancer and have developed model systems of estrogen deprivation that are sensitive [4-6] or resistant to the apoptotic actions of estrogen [7]. In particular, we have previously reported the development of an estrogen deprived breast cancer cell line, MCF-7:5C, which undergoes estradiol-induced apoptosis after two days of treatment via the mitochondrial pathway [8]. In contrast, we have another estrogen deprived breast cancer cell line, MCF-7:2A, which appears to be resistant to estradiol-induced apoptosis [7]. We are studying resistance to estrogen induced apoptosis because clinical experience tells us that only 30% of patients respond to estrogen induced apoptosis once exhaustive antihormonal therapy occurs [9]. An important goal would be to see whether the apoptotic effect of estrogen can be enhanced in antihormone resistant cells. This new targeted approach to the treatment of metastatic breast cancer could open the door to novel approaches to treatmentw ithdr ugc ombinations.

L-buthionine sulfoximine (BSO) is a specific γ-glutamylcysteine synthetase inhibitor that blocks the rate-limiting step of glutathionine (GSH) biosynthesis and in doing so depletes the intracellular GSH pool in both cultured cells and in whole animals [10]. GSH is a water-soluble tripeptide composed of glutamine, cysteine, and glycine. Reduced glutathione is the most abundant intracellular small molecule thiol present in mammalian cells and it serves as a potent

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intracellular antioxidant protecting cells from toxins such free radicals [11, 12]. Changes in GSH homeostasis have been implicated in the etiology and progression of a variety of human diseases, including breast cancer [13]. In particular, studies have shown that elevated levels of GSH prevent apoptotic cell death whereas depletion of GSH facilitates apoptosis [10, 14]. BSO depletes cellular GSH [10] and sensitizes tumor cells to apoptosis induced by standard chemotherapeutica gents[ 15,16] .

Apoptosis or programmed cell death is required for normal development and tissue homeostasis in multicellular organisms. Deregulation of apoptosis is fundamental to many diseases, such as cancer, stroke, heart disease, neurodegenerative disorders, and autoimmune disorders [17]. There are two main pathways for apoptosis, namely the extrinsic receptor mediated pathway and the intrinsic mitochondria-mediated pathway [18, 19]. Components of the extrinsic pathway include the death receptors FasR/FasL, DR4/DR5, and tumor necrosis factor (TNFR) [20] whereas the intrinsic pathway centers on the Bcl-2 family of proteins which comprises both proapoptotic proteins, such as Bax, Bak, and Bid  and antiapoptotic proteins, such as Bcl-2 and Bcl-xL [18, 19]. The Bcl-2 family proteins regulate apoptosis by altering mitochondrial membrane permeabilization which leads to the release of apoptogenic factors such as cytochrome c, procaspases, and apoptosis inducing factor (AIF). In particular, Bcl-2 and Bcl-xL inhibit apoptosis by maintaining mitochondrial membrane integrity whereas Bax and Bak facilitate apoptosis by initiating the loss of outer mitochondrial integrity [21]. Apart from its action on the mitochondria, there is also evidence that Bcl-2 possesses antioxidant property. Bcl-2 overexpression increases cellular GSH level which is associated with increased resistance to chemotherapy-induced apoptosis [22, 23] whereas GSH depletion restores apoptosis in Bcl-2 expressingc ells[ 16].

Based on microarray studies we found that the antihormone resistant MCF-7:2A cells express markedly elevated levels of glutathione synthetase (GS) and glutathione peroxidase 2 (GPx2); two enzymes that are involved in glutathione synthesis, which suggests that resistance to estrogen-induced apoptosis might be due to elevated levels of GSH present in these cells. If indeed MCF-7:2A cells do possess high levels of GSH, then it is possible that the use of BSO, as a single agent, might be able to sensitize these cells to estrogen-induced apoptosis. As mentioned before, there is current clinical interest in using low dose estradiol therapy to treat antihormone resistant breast cancer [24] however only a minimal 30% of patients respond to this therapeutic strategy. A combination of BSO and estradiol could possibly be used to improve the efficacy of estradiol as an apoptotic agent if glutathione depletion is fundamental to tumor cell survival. We have addressed the hypothesis that by altering glutathione levels we may be able to enhance apoptosis to estrogen and have employed BSO as our agent of choice because of earlier work clinically which may provide a foundation for subsequent clinical trials.

In the present study, we show that depletion of cellular GSH by BSO sensitizes antihormone-resistant MCF-7:2A cells to estradiol-induced apoptosis which is mediated, in part, by the mitochondrial pathway and also activation of the JNK signaling pathway. We further show that BSO, either alone or in combination with estradiol, causes tumor regression of MCF-7:2A cells *in vivo*.

## Materialsan dM ethods

### Celll inesan dR eagents

The MCF-7 human breast cancer cell line was obtained from Dr. Dean Edwards (University of Texas, San Antonio, TX) and was maintained in phenol red RPMI 1640 medium supplemented with 10% fetal bovine serum , 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1X non-essential amino acids and bovine insulin at 6 ng/mL. The clonal cell line, MCF-7:2A, was derived by growing MCF-7 cells in estrogen-free media for more than 1 year, followed by two rounds of limiting dilution cloning [7]. These cells were grown in phenol red-free RPMI 1640 medium supplemented with 10% 4X dextran-coated, charcoal-treated FBS (SFS). All reagents for cell culture were obtained from Invitrogen. DL-Buthionine sulfoximine (BSO) and 17 beta-estradiol ($E_2$) were from Sigma, rhodamine 123 (Rh123) was from Molecular Probe,.LY294002a ndS P600125w ere fromC albiochem.

### Westernb lotan alysis

The antibodies used for Western blotting included those against SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), caspase-7, caspase-9, phospho Bcl-2 (Ser70), and PARP (Cell Signaling Technology), cytochrome *c* and β-actin (Sigma), cytochrome oxidase subunit IV (Cox IV; Invitrogen), Bax, Bcl-2, and Bcl-xL (Santa Cruz Biotechnology). Western blotting analysis waspe rformed aspr eviouslyde scribed[ 8].

### CellP roliferationA ssays

Proliferation assay was performed as previously described [8]. Briefly, MCF-7 and MCF-7:2A cells were seeded in estrogen-free RPMI media containing 10% SFS at a density of $2x10^4$ cells

per well in 24-well plates. After 24 hours, cells were treated with the respective drugs for 2, 5, and 7 days with retreatment on alternate days. The DNA content of the cells was determined as previously described [25] using a Fluorescent DNA Quantitation kit (Bio-Rad). For each analysis, six replicate wells were used, and at least three independent experiments were performed.

**Detectionof ap optosisb yA nnexinV s taining**

The Annexin V-FITC labeled Apoptosis Detection Kit I (Pharmingen) was used to detect and quantify apoptosisb yf lowc ytometry,a ccordingt ot hem anufacture'si nstructions.

**TUNELs taining forap optosis**

Apoptosis was also determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay using an *in situ* cell death detection kit, POD (Roche Molecular Biochemicals), according to the manufacturer's instructions. Briefly, fixed cells were washed, permeabilized, and then incubated with 50 µL of terminal deoxynucleotidyl transferase end-labeling cocktail for 60 min at 37 °C in a humidified atmosphere in the dark. For signal conversion, slides were incubated with 50 µl of converter-POD (anti-fluorescein antibody conjugated with horse-radish peroxidase) for 30 min at 37 °C, rinsed with PBS, and then incubated with 50 µl of DAB substrate solution for 10 min at 25 °C. The slides were then rinsed with PBS, mounted under glass coverslips, and analyzed under a light microscope (Inverted NikonT E300).

**Glutathioneas say**

Total cellular glutathione was measured using the Total Glutathione Colorimetric microplate assay Kit (Oxford Biomedical Research), according to the manufacture's protocol. Cells were plated at 0.5 x $10^6$/well of a six-well plate and allowed to recover overnight. After appropriate treatments, cells were washed in PBS and then lysed in 100–150 µl of buffer (100 mM NaPO$_4$, 1 mM EDTA, pH 7.5) containing 0.1% Triton X-100 and frozen at –80°C until analysis. To measure total glutathione, proteins were precipitated with sulfosalicylic acid at a final concentration of 1%. Samples were then spun for 10 min in a microcentrifuge to pellet proteins, and supernatant was diluted 1:20 in buffer before being measured. For all measurements, 50-µl triplicates of each sample were used for glutathione determination. The GSH level was obtained byc omparingw itht heG SHs tandardsa ndr epresented asnm ol/mgof pr otein.

**MitochondrialT ransmembrane Potential( ΔΨ$_m$)an dc ytochromec release**

Changes in the mitochondrial membrane potential (ΔΨm) were examined by monitoring the cells after staining with rhodamine 123. Briefly, estradiol plus BSO-treated MCF-7:2A cells were washed twice with PBS and incubated with 1 µg/mL rhodamine 123 at 37 °C for 30 min. Cellsw eret hen washedt wicew ithP BS,a ndR h123i ntensity wasd eterminedb y flowc ytometry. Cells with reduced fluorescence were counted as having lost some of their mitochondrial membranepot ential.

For cytochrome *c* release assays, cells were lysed in lysis buffer [10 mmol/L HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; pH 7.5), 10 mmol/L KCl, and 1 mmol/L EDTA] with protease inhibitor cocktail (Sigma), frozen and thawed thrice, and spun at 2,000 x *g* for 5 min. The supernatants were centrifuged at 10,000 x *g* for 15 min at 4°C, and the

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mitochondrial pellets were dissolved in SDS sample buffer, subjected to 15% SDS-PAGE, and analyzed by immunoblotting with monoclonal antibodies against cytochrome *c* and cytochrome oxidases ubunitI V( COXIV).

**RNAi solationan dQ uantitativer eal-time PCR**

Total RNA was isolated using TRI reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of RNA was reverse transcribed to cDNA using the SuperScript II RNase H-reverse transcriptase system (Invitrogen, Life Technologies, Carlsbad, CA). Aliquots of the cDNA were combined with the SYBR green kit and primers, and assayed in triplicate by real-time quantitative PCR using a GeneAmp® 5700 Sequence detection system (Applied Biosystems). Quantitation was done using the comparative CT method with 18S rRNA as the normalization gene, as previously described [8]. GS and GPx 2 primers were designed using Primer Express™ software following manufacturer's guidelines. Primers were synthesized by Applied Biosystems. Quantitative PCR was performed using the following conditions: 40 cycles; denaturation at 95C for 15 sec, annealing at 63C for 1 min, and polymerization at 72C for 1 min. Primer sequences were: GS forward:CACCAGCT GGGGAAGCATCT; reverse: GGTGAGGGGAAGAGCGT GAA, GPx 2 forward: TTG ATT AAG GCT TTC TTT GGT AGG;r everse:T TTC AAT AAA TCA GG TCCC AGG .

**Inhibitiono fM CF-7:2AC ellT umorigenesisb yB SOi nN udeM ice**

4-5 week-old female CrTac:NCr-Foxn1nu athymic mice were purchased from Taconic (Germantown, NY). Animal experiments were conducted at the Fox Chase Cancer Center (Philadelphia, PA). The research protocol was approved, and mice were maintained in

accordance with institutional guidelines of the Fox Chase Cancer Center Animal Care and Use Committee. Mice were acclimatized to the animal facility for 1 week before they received injections of MCF-7:2A human breast cancer cells: $2 \times 10^7$ cells were resuspended in 100 µL PBS (Collaborative Biomedical Products, Bedford, MA) and were bilaterally injected into the mammary fat pads of 20 ovariectomized mice. Tumors were allowed to develop for 20 days until they reached a mean cross sectional area of 0.32 cm$^2$, when treatment was initiated with placebo (saline), E2 (0.3 cm capsule), BSO (4mmol/kg weight), or BSO (4mmol/kg weight) plus E2 (0.3 cm capsule) for an additional 7 days. For the estradiol treatment, 0.3 cm silastic estradiol capsules (Baxter HealthCare, Mundeleine, IL) were implanted subcutaneously in the mice. These capsules produced a mean serum estradiol level of 83.8 pg/mL [26], to achieve postmenopausal serum levels of estradiol. BSO was dissolved in saline and was administered i.p. daily for 7 days. The cross-sectional tumor area was calculated by multiplying the length (*l*) by the width (*w*) by $\pi$ and dividing the product by four (i.e., $lw\pi/4$). Animals were given food and water *ad libitum*. Mice from each group (n = 5) were sacrificed at the conclusion of the experiment and immunohistochemical analysisw asp erformed.

**TissueP reparationan dI mmunohistochemistry**

Tumors from mice treated with placebo, E2, BSO, or BSO plus E2 were excised and fixed in 10% formalin, embedded in paraffin wax blocks and sectioned. Subsequently, sections of the blocks were stained with hematoxylin and eosin (H&E) or Ki67 antibody (1:500 dilution, Santa CruzB iotechnology)b yt hepa thology core facility at FoxC haseC ancerC enter.

**Statisticalan alysis**

Statistical analysis was performed using Student's *t* test, and a *P* value of <0.05 was considered significant. Data are expressed as the mean ± SE. The mean value was obtained from at least threei ndependente xperiments.

**Results**

**Estrogend eprivationi ncreasesgl utathionel evelsi nM CF-7:2Ab reast cancer cells**

Elevated glutathione levels and the activity of its related enzymes have been characterized as one of the factors which could render breast cancer cells resistant to apoptosis. We have previously shown that MCF-7:2A breast cancer cells are resistant to estrogen-induced apoptosis [7], therefore we measured glutathione levels in these cells along with parental MCF-7 cells. Figure 1a showed that glutathione levels were significantly higher in MCF-7:2A cells (11.9 µM/mg protein) compared to MCF-7 cells (7.8 µM/mg protein) and treatment with BSO (100 µM), an inhibitor of glutathione synthesis, for 24 hours depleted glutathione content by ~55% and 68% in MCF-7 and MCF-7:2A cells, respectively. It is worth noting that glutathione levels were consistently elevated in MCF-7:2A cells up to 7 days and the inhibitory effect of BSO persistedt hroughoutt hati ncubationpe riod( datan ots hown).

We next examined whether the expression of glutathione-related enzymes was altered in these cells. Using quantitative real-time PCR, we found a 6-fold increase in glutathione synthetase (GS) expression and a 40-fold increase in glutathione peroxidase 2 (GPx2) expressions in MCF-7:2A cells compared to parental MCF-7 cells (Figure 1b). Western blot analysis also showed a marked increase in GS protein level in MCF-7:2A cells compared to parentalM CF-7c ells( Figure1b ,r ightpa nel).

**BSOe nhancest heap optotice ffectof 17 β-estradioli nM CF-7:2A cells**

We next examined whether depletion of glutathione levels by BSO sensitizes MCF-7:2A cells to estrogen-induced apoptosis. For proliferation assays, MCF-7 and MCF-7:2A cells were seeded in estrogen-free media, and after 24 hours, were treated with 100 µM BSO, 1 n*M*

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estradiol (E$_2$), or 100 μM BSO plus 1 n*M* estradiol for 2, 5, and 7 days. Figure 2a shows that the growth of parental MCF-7 cells was stimulated 7-fold over the control cells by 1 n*M* estradiol during the course of the 7-day assay and that treatment with BSO, either alone or in combination with estradiol, did not significantly alter the growth of these cells. In contrast, MCF-7:2A cells treated with the combination of 100 μM BSO and 1 n*M* estradiol showed a significant time-dependentd ecreasei n cellg rowthr elativet o cellst reatedw ith either estradiolor BSOa lone.T he cell killing effect of BSO and estradiol was observed as early as 48 hours after treatment and persisted over the time course of the experiment with maximum cell death at the 7-day time point.

Based on the above finding, we next determined whether MCF-7:2A cells underwent apoptotic cell death upon BSO and estradiol treatment. We performed TUNEL assay, which detects the fragmentation of DNA which is characteristic of cells undergoing apoptotic cell death. As shown in Figure 3a, the percentage of TUNEL-positive cells significantly increased with the combination of BSO and estradiol but not with estradiol or BSO alone. After treatment with BSO and estradiol (96 hours), as many as 53% of cells displayed TUNEL-positive staining, whereas, only 1% of the control cells and 5% of the estradiol treated cells were TUNEL-positive. BSO-treated cells looked similar to control cells (data not shown). As expected, parental MCF-7 cells showed very little TUNEL-positive staining in the presence of estradiol alone or BSO plus estradiolc ombined( Figure2b , *toppane l*),t husi ndicating al ackof apoptosisi nt hesec ells.

To further substantiate the apoptotic effect of BSO and estradiol in MCF-7:2A cells, annexin V-PI immunostaining was performed by flow cytometry. Figure 3b shows that in the BSO plus estradiol-treated group, approximately 55.6% of cells stained positive for annexin V whereas in the control group and estradiol-treated group, ~7.4% and ~12.6%, respectively, of cells stained

positive for annexin V. For the BSO-treated group, only 4.7% of cells stained positive for annexin( datanot s hown).

**Role of the Mitochondrial Pathway in BSO plus Estradiol-Induced Apoptosis in MCF-7:2A Cells**

To examine the role of the mitochondrial pathway in BSO plus estradiol-induced apoptosis, Western blot analyses was used to measure Bax, Bcl-2, phosphorylated Bcl-2, and Bcl-xL protein levels in MCF-7:2A cells following treatment with 1n$M$ estradiol alone, 100 μM BSO, or BSO plus estradiol for 48 hours. We found that Bcl-2, phospho-Bcl-2, and Bcl-xL protein levels were almost completely reduced in MCF-7:2A cells treated with BSO plus estradiol compared to control, BSO, or estradiol alone. In addition, a marked increase in Bax expression was also observed in MCF-7:2A cells following BSO plus estradiol combined treatment (Figure 4a). In contrast, similar experiments performed with parental MCF-7 cells showed that BSO plus estradiol slightly increased Bcl-2 and phospho-Bcl-2 protein levels in these cells with a more dramatic effect observed with estradiol alone (Figure 4a). It is worth noting that in MCF-7:2A cells endogenous levels of Bcl-2 and phosphorylated Bcl-2 were markedly elevated compared to parental MCF-7 cells. This finding is consistent with previous reports which show that overexpression of Bcl-2 increases glutathione levels and inhibits mitochondrial dysfunction and cellde athe licitedb y glutathione-depletingr eagents[ 27].

Although estradiol, as an individual treatment, did not significantly induce apoptosis in MCF-7:2A cells, it did decrease Bcl-2 protein level in these cells. We therefore tested whether siRNA knockdown of Bcl-2 expression would sensitize MCF-7:2A cells to estradiol-induced apoptosis. Expression of Bcl-2 following knockdown was analyzed by western blotting. As

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expected, Bcl-2 protein levels were significantly reduced following transfection of MCF-7:2A cells with Bcl-2 siRNA compared to control siRNA (Figure 4b, top panel). Using annexin V staining, we found that apoptosis was increased by 20% in Bcl-2 siRNA transfected cells compared with cells transfected with the control siRNA (Figure 4b, bottom panel), thus suggesting that suppression of antiapoptotic factors such as Bcl-2 has the ability to partially sensitizehor mone-independentM CF-7:2A cellst oa poptosis.

We next examined mitochondrial membrane integrity using RH123 retention assay. Cells weret reatedw ithnot hing (control), estradiol, BSO,or  BSOpl use stradiolf or48hour  s.F igure4 c shows that BSO plus estradiol treatment reduced RH123 fluorescence in MCF-7:2A cells by ~50% compared to control, whereas, estradiol or BSO, as individual treatments, did not significantly alter RH123 retention levels in these cells. BSO plus estradiol also enhanced cytochrome *c* release in MCF-7:2A cells. Figure 4d shows that in the control cells, cytochrome *c* was detected primarily in the mitochondria and was undetectable in the cytosol; however, in the presence of BSO plus estradiol (48 hours), all of cytochrome *c* was observed in the cytosol. BSO or estradiol, as individual treatments, did not significantly alter mitochondrial release of cytochrome *c*. The translocation of cytochrome *c* from the mitochondria to the cytosol following BSO plus estradiol treatment coincided with cleavage of caspase 7 and poly(ADP-ribose) polymerase (PARP) (Figure 4e), which is a molecular signature of apoptosis. Cleavage of PARP and caspase 7 was blocked by the pan-caspase inhibitor z-VAD (data not shown). There was however no cleavage of caspase 9 in MCF-7:2A cells following BSO plus estradiol treatment (datanot s hown).

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**The apoptotic effect of BSO and estradiol in MCF-7:2A cells is regulated, in part, by JNK signaling**

Emerging evidence supports a role for JNK in stress-induced mitochondrial apoptotic pathways in a variety of cell systems [28]. Therefore, we examined the possible involvement of c-Jun/JNK pathway in BSO plus estradiol-induced apoptosis in MCF-7:2A cells. JNK activation was determined by western blot analysis after 48-h treatment of cells with BSO plus estradiol. A profound induction of the p54 and p46 isoforms of phosphorylated JNK as well as a significant increase in phospho-c-Jun and c-Jun were observed in MCF-7:2A cells treated with BSO plus estradiol compared to BSO alone or control (Figure 5a). Interestingly, treatment with estradiol alone also significantly increased phosphorylated JNK in MCF-7:2A cells. We also found that pretreatment of MCF-7:2A cells with the JNK inhibitor, SP600125 (20 μM) markedly reduced thea poptotice ffectof  BSOpl use stradioli nt hesec ells( Figure5 b).O verall,t heser esultss uggest a possible involvement of the c-Jun/JNK signaling pathway in BSO plus estradiol-induced apoptosisi nM CF-7:2Ac ells.

**BSOi nhibitst hegr owthof M CF-7:2A cells *inv ivo***

To determine whether the effect of BSO plus estradiol was relevant *in vivo*, we used a xenograft model in which MCF-7:2A cells were injected into CrTac:NCr-Foxn1nu athymic mice (n=20). After 20 days postinjection, tumors grew to a mean crossectional area of 0.30 cm$^2$ and mice were randomized to four groups; placebo (saline), estradiol, BSO, or the combination of BSO plus estradiol, as described in materials and methods. After 7 days of treatment, tumor growth was reduced by 25% in mice treated with estradiol alone whereas in the BSO and BSO plus estradiol group tumor growth was reduced by 40% and 60%, respectively, compared to the

placebo group which showed a 7% increase in growth (Figure 6a). Interestingly, we found that BSO *in vitro* had a relatively small effect on growth, however, *in vivo* its effect was very pronounced, thus suggesting the possibility of altered glutathione metabolism *in vivo*. We performed histology on tumors taken from placebo, estradiol, BSO, and BSO plus estradiol groups at day 27. H&E staining of the BSO plus estradiol-treated tumors revealed less tumor cells and more intercellular matrix, significantly less mitoses, chromatin clumping and dark staining which are associated with apoptosis, and enhanced abnormalities in shape and size, compared to tumors from placebo or BSO or estradiol-treated groups (Figure 6b). We also characterized the proliferative status of these cells by staining tumors for the expression of Ki67, a marker of cell proliferation. We observed a 32% decrease ($P < 0.001$) in the number of Ki67 stained tumors from the BSO plus estradiol-treated group and a 21% decrease in the BSO-treated group compared to the placebo group whereas estradiol treatment caused an 8% increase in Ki67 staining (Figure 6c). Overall, these data show that BSO either alone or in combination with estradiol, reduces tumor growth by possibly increasing apoptosis and decreasing the proliferation oft umorc ells.

**Discussion**

In the current study, we investigated whether suppression of the antioxidant glutathione by BSO has the ability to sensitize antihormone resistant MCF-7:2A breast cancer cells to estradiol-induced apoptosis. Our results showed that glutathione levels and the enzymes involved in its synthesis, glutathione synthetase and glutathione peroxidase, were significantly elevated in MCF-7:2A cells compared to parental MCF-7 cells and that suppression of glutathione by BSO sensitized these cells to estrogen-induced apoptosis *in vitro* and *in vivo*. The BSO-mediated estradiol-induced apoptosis was associated with a marked decrease in the expression of antiapoptotic Bcl-2 and Bcl-xL proteins and a significant increase in proapoptotic Bax protein. It is worth noting that high-dose estrogen was generally considered the endocrine therapy of choice for postmenopausal women with breast cancer prior to the introduction of tamoxifen, however, due to undesirable side effects, the use of high-dose estrogen was largely abandoned [29]. Here, we show that the killing effect of estradiol in antihormone resistant cells can be achieved at physiological concentrations when it is combined with non-toxic concentrations of BSO. Our present findings are consistent with previous studies which have shown that the cytotoxicity of a number of chemotherapeutic drugs, including melphalan [30], doxorubicin [31], and bleomycin [32],a res ignificantlye nhancedw hen glutathionei sde pletedb y BSO.

An important target of BSO plus estradiol-induced apoptosis appears to be Bcl-2 whose protein expression was dramatically decreased in MCF-7:2A cells following glutathione depletion. Previous studies have shown that Bcl-2 functions as an antioxidant to block apoptosis and that Bcl-2 protein levels and glutathione intracellular concentration is coordinately regulated with a decrease in either favoring cell death [23, 33]. It is believed that one mechanism by which Bcl-2 may function as an antioxidant is through up-regulation of glutathione, leading to rapid

detoxification of reactive oxygen species and inhibition of free radical-mediated mitochondrial damage. Bcl-2 also has the ability to shift the entire cellular redox potential to a more reduced state which is independent of its effect on glutathione levels [33]. It is worth noting that glutathione levels and Bcl-2 protein expression were significantly elevated in MCF-7:2A cells compared to parental MCF-7 cells. In phase I trials [34, 35], the concentration of BSO in blood has been shown to reach 0.5 to 1 mM, whereas, in mice [36, 37] the concentration has been estimatedt obe 5t o6m Mf ollowing an *inv ivo*t reatmentof 4m mol/kg. Inour s tudy,w es howed that 100 μM BSO decreased glutathione concentrations by ~60% after 24 hours and that BSO enhanced the apoptotic effect of estradiol in MCF-7:2A breast cancer cells as early as 48 hours after treatment. Interestingly, treatment with BSO alone did not cause apoptosis in MCF-7:2A cells, indicating that glutathione depletion alone may not trigger apoptosis in these cells. This finding is consistent with previous studies by Mirkovic et al [38] which showed that inhibition of glutathioneb y BSOdi dn oti ncreases usceptibility ofm ousel ymphoma cellst or adiation-induced apoptosis even under conditions where glutathione levels were lowered by 50%. Other groups have made similar observations using BSO [39]. One possible explanation for this apparent contradiction might be the fact that BSO does not lower glutathione levels in mitochondria as effectively as it does in the cytoplasm [40]. Mitochondrial glutathione concentrations are regulated and have been implicated in apoptotic cell death [41], hence, it would be of interest to evaluate relative glutathione concentrations in the mitochondrial matrix of MCF-7:2A cells following treatment with BSO either alone or in combination with estradiol. Another possibility could be that cellular thiols other than glutathione may play important roles in regulating apoptosis [39]. The flavoprotein thioredoxin has been shown to be upregulated in several human tumors and is implicated in both cancer cell growth and apoptotic resistance [42]. However, it is

not known whether Bcl-2 or other apoptotic regulators can influence the levels of thioredoxin or whethers uchm odulationm ay contributet or esistancei nhum ant umorc ells.

Apart from Bcl-2, we also found that proapoptotic Bax protein was markedly increased in MCF-7:2A cells by the combination of BSO plus estradiol and this induction coincided with a loss of mitochondrial membrane integrity and cytochrome *c* release. Bax is normally found as a monomer in the cytosol of nonapoptotic cells and it oligomerizes and translocates to the outer mitochondrial membrane in response to apoptotic stimuli and induces mitochondrial membrane permeabilization and cytochrome *c* release [19]. In MCF-7:2A cells, Bax protein was induced as early as 24 hours after BSO plus estradiol treatment (Figure 4) and suppression of Bax expression using siRNA was able to partially reverse the apoptotic effect of the combination treatment (data not shown). The induction of Bax coincided with cytochrome c release from the mitochondria into the cytosol which was followed by activation of caspase 7 and PARP cleavage. Iti sw orthnot ingt hatpr etreatmentof c ellsw itht heuni versalc aspasei nhibitorz -VAD almost completely blocked the apoptotic effect of BSO plus estradiol. It is worth noting that antiapoptotic Bcl-2 and Bcl-xL proteins were also markedly decreased in MCF-7:2A cells following the combination treatment of BSO plus estradiol (Figure 4) and overexpression of Bcl-xL partially blocked the apoptotic effect of BSO plus estradiol (data not shown). This finding is important because there is evidence that suggests that the ratio rather than the amount of antiapoptotic versus proapoptotic proteins determines whether apoptosis will proceed [43]. Thus, it is reasonable to suggest that the apoptotic effect of BSO plus estradiol is mediated, in part, by the mitochondrial pathway through their ability to alter the ratio between proapoptotic and antiapoptoticpr oteinsi nt argetc ells.

In addition to the mitochondrial pathway, BSO plus estradiol appears to induce apoptosis, in part, through activation of the c-Jun N-terminal kinases (JNK) signaling pathway. JNKs are a group of MAPKs that bind the NH2-terminal activation domain of the transcription factor c-jun and phosphorylate c-jun on amino acid residues Ser-63 and Ser-73 [44]. JNKs are stimulated by multiple factors including cytokines, DNA-damaging agents, and environmental stresses and are important in controlling programmed cell death or apoptosis. The inhibition of JNKs has been shown to enhance chemotherapy-induced inhibition of tumor cell growth, suggesting that JNKs may provide a molecular target for the treatment of cancer [44]. We found that JNK activation (as measured by the increased levels of phospho-JNK1/2 and the JNK substrate phospho-c-Jun) correlated well with BSO plus estradiol-induced apoptosis in MCF-7:2A cells and pharmacologic disruption of this pathway using the JNK inhibitor SP600125 significantly attenuated this effect. The exact mechanism by which JNK promotes apoptosis is not known; however, the phosphorylation of transcription factors such as c-jun and p53, as well as pro- and anti-apoptotic Bcl-2 family members [45] has been suggested to be of importance. It is worth noting that treatment with BSO plus estradiol markedly increased phosphorylated c-jun in MCF-7:2A cells and decreased phosphorylated Bcl-2 in these cells. These findings thus suggest that BSO plus estradiol might mediate their apoptotic effect, in part, through activation of JNK.

**Conclusion**

We have demonstrated that glutathione depletion by BSO sensitizes hormone-resistant MCF-7:2A human breast cancer cells to estradiol-induced apoptosis *in vitro* and *in vivo*. This laboratory finding has important clinical implications; particularly for the use of estrogen deprivation as long-term therapy, and it suggest that, if and when resistance develops, a strategy

of treatment with estrogen combined with BSO may be effective in sensitizing resistant cells to apoptosis. It is worth noting that recently, Lonning and coworkers [9] reported a 33% complete response (i.e. stable disease) with high dose diethylstilbestrol (DES) in post-menopausal patients with advanced breast cancer who were heavily pre-treated with endocrine agents. However, sixty-seven percent of the patients showed partial or no response [9] so the key to future clinical progress in the treatment of antihormone resistant breast cancer is to improve current treatment strategies. We are currently evaluating the optimal dose of daily estradiol therapy to reverse antihormonal resistance [4] but the goal is to enhance the estradiol-induced apoptotic response. Our present findings suggest that BSO is indeed capable of enhancing the apoptotic effect of estradiol in antihormone resistant breast cancer cells. It is worth noting that a phase I study of BSO administered with the anticancer drug melphalan showed that continuous-infusion of BSO was relatively nontoxic and resulted in depletion of tumor glutathione [35, 46]. Thus it is possible that future clinical studies of BSO infusions combined with low dose estrogen hold the promise of improving disease control for patients with antihormone resistant ER positive metastaticbr east cancer.

## Abbreviations

BSO = L-buthionine sulfoximine, E2 = estradiol, ER = estrogen receptor, FBS = fetal bovine serum, GCS = glutamylcysteine, GPx2 = glutathione peroxidase, GS = glutathione synthetase, GSH = glutathione, H & E = hematoxylin and eosin, JNK = c-jun N-terminal kinase, RH123 = rhodamine 123, SFS = dextran coated charcoal-treated FBS, TUNEL = terminal deoxynucleotidylt ransferase-mediateddU TPe nd labeling.

## Competingi nterests

Thea uthorsde claret hatt heyh aveno competingi nterests.

## Authors'c ontributions

JSLW designed and coordinated the studies, analyzed the data and interpreted the results, generated the figures, and wrote and revised the manuscript. HK performed the cell proliferation assays and the Western blots. CW performed the glutathione assay. RP and JP performed the animal experiments. AJK performed the immunohistochemistry. VCJ is the PI of the laboratory inw hicha ll experiments werec onducted andi st her ecipientof t he grantt hatpa rtially fundedt he project. He was instrumental in revising the manuscript. All authors read, assisted in revision and approvedt he finalm anuscript.

## Acknowledgments

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**FigureL egends**

**Figure 1.** Intracellular glutathione levels in wild-type MCF-7 cells and antihormone-resistant MCF-7:2A breast cancer cells. (a) MCF-7 and MCF-7:2A cells were seeded at 2 x 10$^6$ cells per 100 mm culture plates in phenol red RPMI media containing 10% FBS and phenol red-free RPMI media containing 10% 4X dextran coated charcoal-treated FBS, respectively, and after 24 hours were treated with nothing (control) (*white columns*) or 100 μM BSO (*black columns*) for 24 hours. Total cellular glutathione was measured using a Glutathione Colorimetric microplate assay kit, as described in materials and methods. *Columns*, mean from three separate experiments; *bars*, SE. *, $P < .005$, with respect to parental MCF-7 control. (b) Quantitative Real-Time PCR of glutathione sythetase (GS) (*top left*) and glutathione peroxidase 2 (GPx2) (*bottom left*) mRNA expression in MCF-7 and MCF-7:2A cells. Western blot analysis of GS proteine xpressioni nM CF-7:2Ac ellsi sa lsos hown( *topr ight*)

**Figure2.** E ffectof BSO pluse stradiolont hegr owthof w ild-typeM CF-7 cellsa nda ntihormone-resistant MCF-7:2A cells. (a) MCF-7 cells were grown in estrogen-free media for 3 days prior to the start of the growth assay. On the day of the experiment, 30,000 cells were seeded in 24-well plates and after 24 hours were treated with < 0.1% ethanol vehicle (control), 1 nM estradiol (E2), 100 μM BSO, or 100 μM BSO plus 1 nM E2 for 7 days. At the indicated time points, cells were harvested and total DNA (ng/well) was quantitated as described in Materials and methods. (b) MCF-7:2A cells were seeded at the same density as MCF-7 cells and were treated similarly. The datar epresentt hem eano ft hreei ndependente xperiments.

**Figure 3**. BSO plus estradiol induce apoptosis in MCF-7:2A cells. (a) TUNEL staining for apoptosis in MCF-7:2A cells following BSO plus E2 treatment for 96 hours were performed as
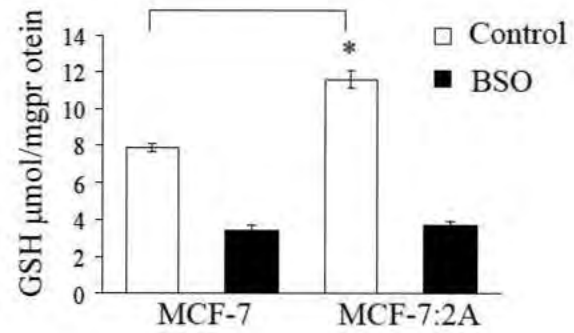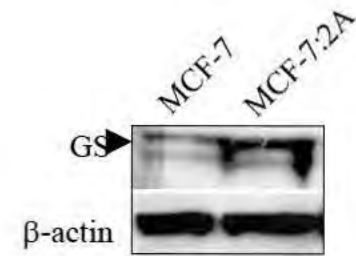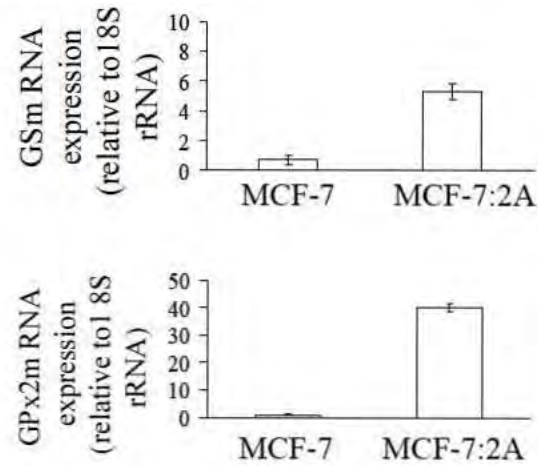
described in Materials and methods. Slides were photographed through brightfield microscope under 100X magnification. TUNEL-positive cells were stained black (white arrows). *Columns* (*right*), mean percentage of apoptotic cells (annexin V-positive cells) from three independent experiments done in triplicate; bars, SEs. (b) Annexin V staining for apoptosis. Cells were seeded in 100 mm plates at a density of 1 x 10$^6$ per plate and after 24 hours were treated with ethanol vehicle (control), 1 nM E2, or BSO plus E2 for 72 hours and then stained with FITC-annexinV a ndpr opidiumi odide( PI)a nda nalyzedb yf lowc ytometry.P I (Pharmingen)w asus ed as a cell viability marker. Representative cytograms are shown for each group. Quantitation of apoptosis( percentof c ontrol)i nt hedi fferentt reatmentg roupsi ss hownon t he *right*.
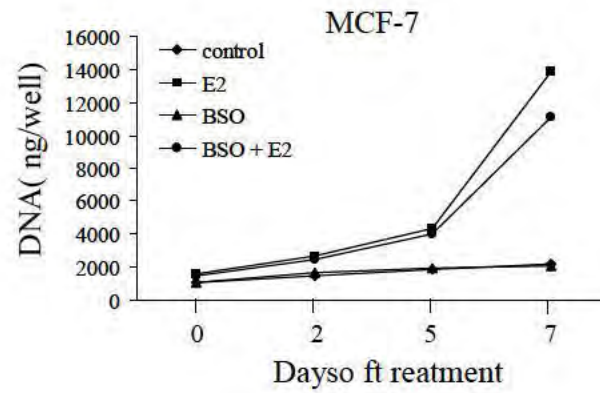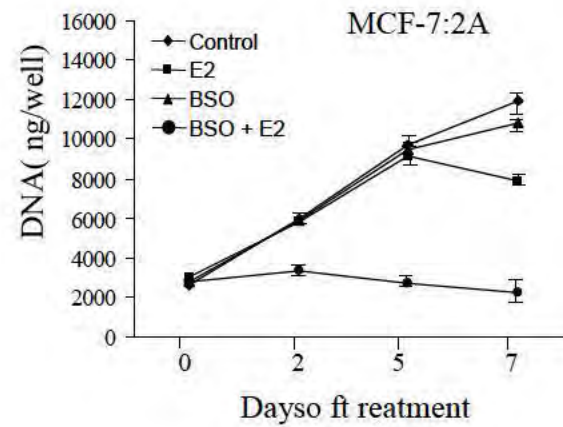
**Figure 4.** Effect of BSO and E2 on Bcl-2 family protein expression and mitochondrial function in MCF-7 and MCF-7:2A cells. (a) Western blot analysis for pBcl-2, Bcl-2, Bcl-x$_L$, and Bax protein expression in parental MCF-7 cells and MCF-7:2A cells following 48 hours of treatment with ethanol vehicle (Control), 1 nM E$_2$, 100 μM BSO, or E2 + BSO. Equal loading was confirmed by reprobing with an antibody against β-actin. (b) siRNA knockdown of Bcl-2 partially sensitizes MCF-7:2A cells to E2-induced apoptosis. Cells were transfected with siRNA-Bcl-2 or siRNA-Con (control) and expression levels of Bcl-2 was determined by immunoblot analysis (*top*). Annexin V staining (*bottom*) showing the effects of siRNA-con and siRNA-Bcl-2 on apoptosis induced by estradiol treatment in MCF-7:2A cells. *, $P < 0.001$. (c) Loss of mitochondrial potential in MCF-7:2A cells was determined by rhodamine 123 (Rh123) retention assay. The percentage of cells retaining Rh123 in each treatment group was compared with untreated control. (d) Cytochrome *c* release from the mitochondria to the cytosol after treatment with E2 alone or BSO and E2 for 48 hours was determined as described in Materials and methods. Anti-COXIV (subunit IV) antibody was used as a control to demonstrate that
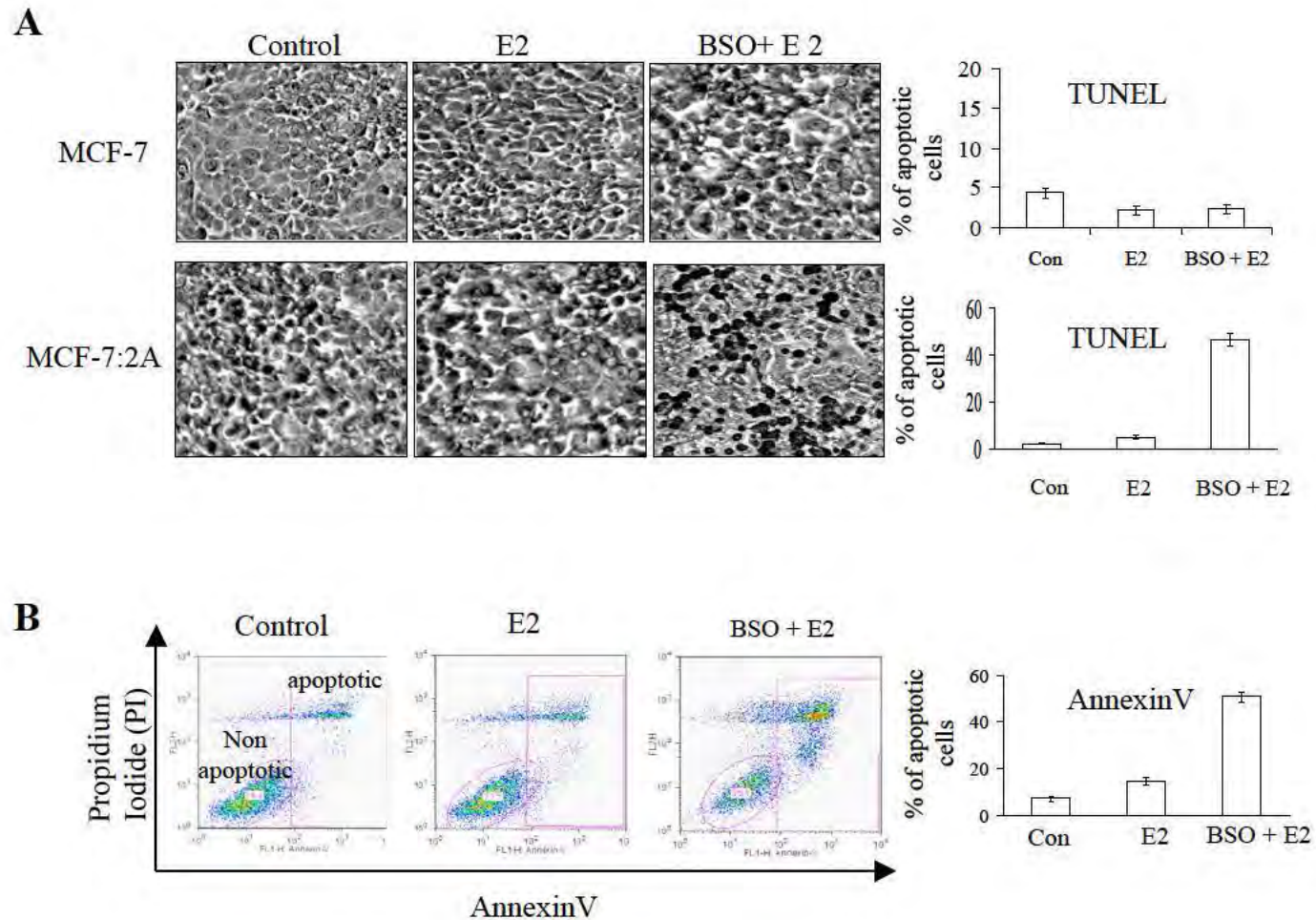
mitochondrial protein fractionation was successfully achieved. **(e)** cleavage of caspase 7 and PARP (72 hours) was assessed by Western blot using specific antibodies. The upper band of caspase 7 represents the full length protein and the lower band (p20, arrow) represents the cleaved activated product; NS, nonspecific. Full length PARP is approximately 116 kDa; cleaved (active) PARP is 85 kDa (arrow). The results are representative of three independent experiments.
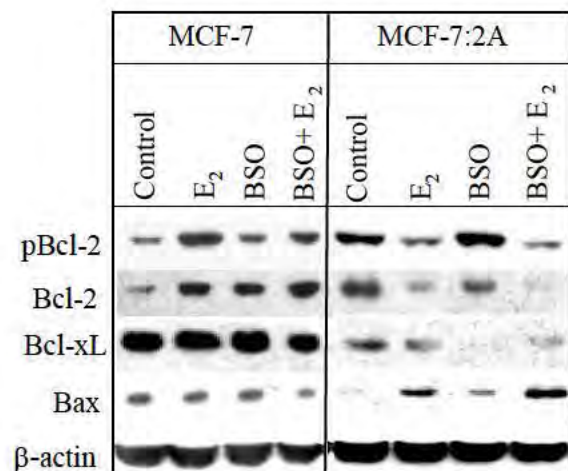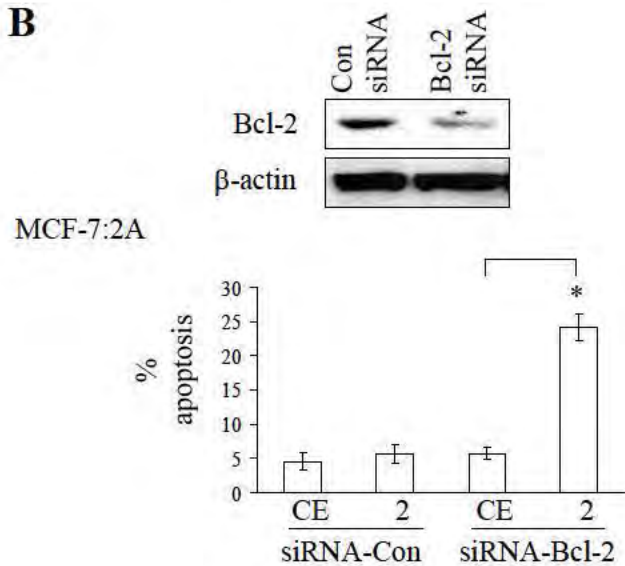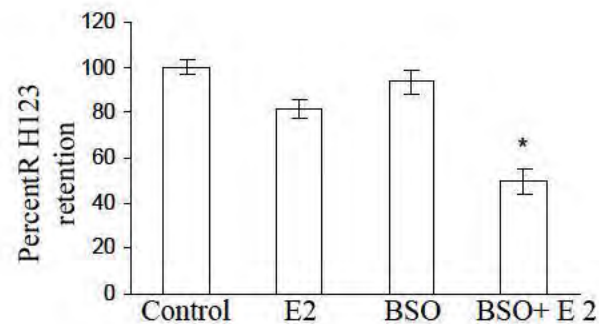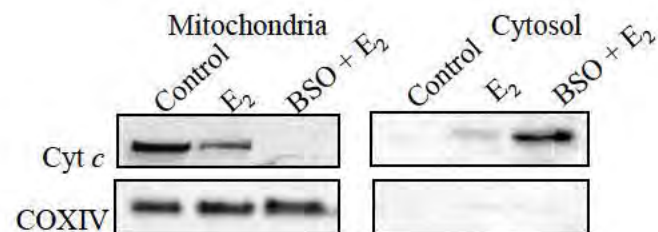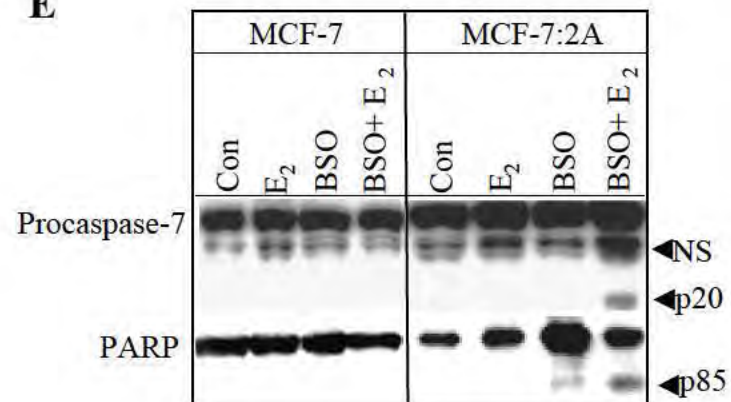
**Figure 5.** Activation of JNK signaling pathway in MCF-7:2A cells in response to BSO and estradiol treatment. (a) MCF-7 and MCF-7:2A cells were treated with ethanol vehicle (control), 1 nM E2 or 100 μM BSO plus E2 for 48 hours and protein levels of phosphorylated JNK, JNK, phosphorylated c-Jun, and c-Jun were analyzed by Western blotting. β-actin was used as a control. (b) Inhibition of JNK activation by SP600125 (SP) partially reverses the apoptotic effect of BSO and estradiol in MCF-7:2A cells. Cells were pretreated with 20 μM SP600125 or vehicle for 24 hours, then further incubated for 48 hours with 1 nM E2, E2 + 100 μM BSO, 20 μM SP, or E2 + BSO + SP and apoptosis was determined by annexin V-PI staining as described in Materials in methods. *Columns*, mean percentage of apoptotic cells from three independent experiments done in triplicate; bars, SEs. ($P < 0.001$, two-tailed $t$ test). (c) Inhibition of the PI3K/Akt signaling pathway inhibits growth and induces apoptosis in MCF-7:2A cells. Cells were pretreated with the PI3K inhibitor LY294002 (LY) for 24 hours, then further incubated with 1 nM E2 or ethanol vehicle (control) for 96 hours (bottom) or 7 days (top). *Columns* (*bottom*), mean percentage of apoptotic cells from three independent experiments done in triplicate;ba rs,S Es.( $P< 0.01$,t wo-tailed $t$ test).
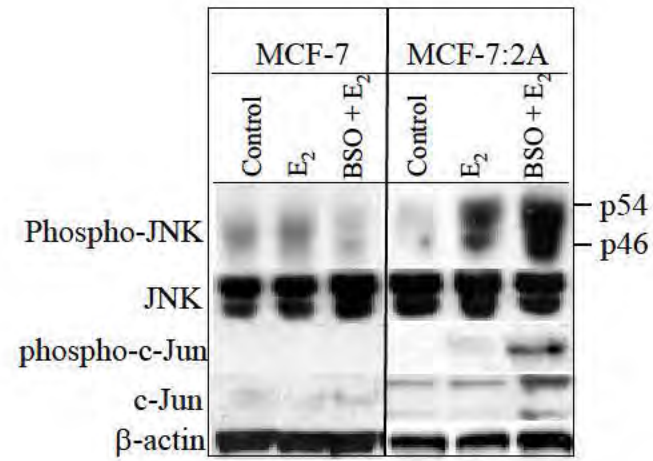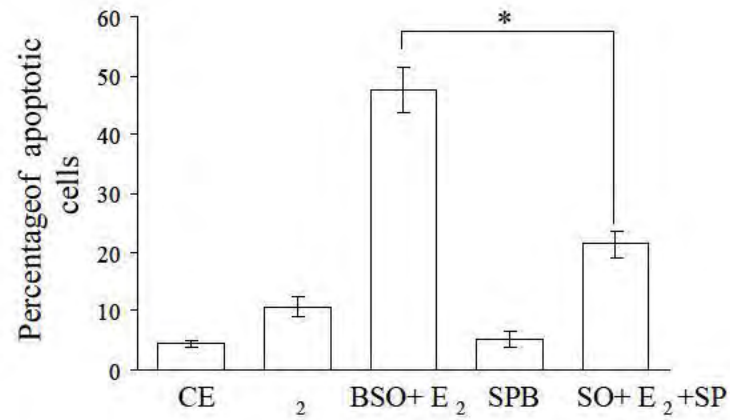
**Figure 6.** BSO inhibits the growth of MCF-7:2A tumors *in vivo*. 4-5 weeks athymic nude mice (n = 20) were injected with MCF-7:2A breast cancer cells and after 20 days when tumors had reached a mean cross sectional area of 0.3 cm$^2$, animals were randomized into 4 groups and were treated with placebo (saline), estradiol (E2), BSO, or BSO plus E2 for 7 days as described in materials and methods. BSO (4 mmol/kg weight) was diluted in saline and was injected i.p. daily. (a) Tumor size was measured everyday and cross-sectional area was calculated by multiplying the length (*l*) by the width (*w*) by π and dividing the product by four (i.e., *lwπ/4*). Data is shown as mean ± SE. *, $P < 0.05$, control group compared with E2 group; †, $P < 0.002$ control group compared with BSO group; §, $P < 0.001$ control group compared with BSO+E2 group. (b) Microscopy of H&E-stained histological sections of MCF-7:2A tumors treated with placebo, E2, BSO, and BSO in combination with E2. (c) Immunohistochemical analysis of the proliferation marker Ki-67 in MCF-7:2A tumors treated with placebo, E2, BSO, or BSO plus E2. Threet of ourt umors per treatment groupw ere analyzed.

A



B

Figure 1

Figure1

**A**



MCF-7

- ◆ control
- ■ E2
- ▲ BSO
- ● BSO + E2

DNA( ng/well)

Dayso ft reatment

**B**



MCF-7:2A

- ◆ Control
- ■ E2
- ▲ BSO
- ● BSO + E2

DNA( ng/well)

Dayso ft reatment

**A**

| | Control | E2 | BSO+ E 2 |
|---|---|---|---|
| MCF-7 | | | |



TUNEL

% of apoptotic cells

Con   E2   BSO + E2

MCF-7:2A

TUNEL

% of apoptotic cells

Con   E2   BSO + E2

**B**

Propidium Iodide (PI)

Control
apoptotic
Non apoptotic

E2

BSO + E2

AnnexinV

AnnexinV

% of apoptotic cells

Con   E2   BSO + E2

**Lewis-Wambi etal**

**Figure3**

Figure 3

**A**

MCF-7 / MCF-7:2A

Control, E₂, BSO, BSO+E₂ (both panels)

pBcl-2
Bcl-2
Bcl-xL
Bax
β-actin

**B**

Con siRNA / Bcl-2 siRNA

Bcl-2
β-actin

MCF-7:2A

% apoptosis

CE, 2 (siRNA-Con); CE, 2 (siRNA-Bcl-2)

**C**

Percent R H123 retention

Control, E2, BSO, BSO+ E 2

**D**

Mitochondria — Control, E₂, BSO + E₂
Cytosol — Control, E₂, BSO + E₂

Cyt c
COXIV

**E**

MCF-7 / MCF-7:2A

Con, E₂, BSO, BSO+ E₂ (both panels)

Procaspase-7 ◀NS
◀p20
PARP ◀p85

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Figure4

Figure 4

**A**



**B**

Figure 5

**A** — Averagec ross sectional area (cm$^2$): C, E2 (*), BSO (†), BSO+E2 (§)

**B** — H&E: Control, E2, BSO, BSO+E 2

**C** — Ki67: Control, E2, BSO, E2+B SO; Ki67 Labelingi ndex: C, E2, BSO (*), BSO+E2 (†); P<0.009, P<.0001

Lewis-Wambi etal

Figure6

Figure 6

# Exemestane's 17-hydroxylated metabolite exerts biological effects as an androgen

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## Abstract

**Aromatase inhibitors (AI) are being evaluated as long-term adjuvant therapies and chemopreventives in breast cancer. However, there are concerns about bone mineral density loss in an estrogen-free environment. Unlike nonsteroidal AIs, the steroidal AI exemestane may exert beneficial effects on bone through its primary metabolite 17-hydroexemestane. We investigated 17-hydroexemestane and observed it bound estrogen receptor $\alpha$ (ER$\alpha$) very weakly and androgen receptor (AR) strongly. Next, we evaluated 17-hydroexemestane in MCF-7 and T47D breast cancer cells and attributed dependency of its effects on ER or AR using the antiestrogen fulvestrant or the antiandrogen bicalutamide. 17-Hydroexemestane induced proliferation, stimulated cell cycle progression and regulated transcription at high sub-micromolar and micromolar concentrations through ER in both cell lines, but through AR at low nanomolar concentrations selectively in T47D cells. Responses of each cell type to high and low concentrations of the non-aromatizable synthetic androgen R1881 paralleled those of 17-hydroexemestane. 17-Hydroexemestane down-regulated ER$\alpha$ protein levels at high concentrations in a cell type – specific manner similarly as 17$\beta$-estradiol, and increased AR protein accumulation at low concentrations in both cell types similarly as R1881. Computer docking indicated that the 17$\beta$-OH group of 17-hydroexemestane relative to the 17-keto group of exemestane contributed significantly more intermolecular interaction energy toward binding AR than ER$\alpha$. Molecular modeling also indicated that 17-hydroexemestane interacted with ER$\alpha$ and AR through selective recognition motifs employed by 17$\beta$-estradiol and R1881, respectively. We conclude that 17-hydroexemestane exerts biological effects as an androgen. These results may have important implications for long-term maintenance of patients with AIs. [Mol Cancer Ther 2007;6(11):2817–27]**

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each cell type to high and low concentrations of the non-aromatizable synthetic androgen R1881 paralleled those of 17-hydroexemestane. 17-Hydroexemestane down-regulated ER$\alpha$ protein levels at high concentrations in a cell type – specific manner similarly as 17$\beta$-estradiol, and increased AR protein accumulation at low concentrations in both cell types similarly as R1881. Computer docking indicated that the 17$\beta$-OH group of 17-hydroexemestane relative to the 17-keto group of exemestane contributed significantly more intermolecular interaction energy toward binding AR than ER$\alpha$. Molecular modeling also indicated that 17-hydroexemestane interacted with ER$\alpha$ and AR through selective recognition motifs employed by 17$\beta$-estradiol and R1881, respectively. We conclude that 17-hydroexemestane exerts biological effects as an androgen. These results may have important implications for long-term maintenance of patients with AIs. [Mol Cancer Ther 2007;6(11):2817 – 27]

## Introduction

The third-generation aromatase inhibitors (AI) anastrozole (Arimidex; refs. 1, 2), letrozole (Femara; refs. 3, 4), and exemestane (Aromasin; refs. 5, 6), by virtue of blocking extragonadal conversion of androgens to estrogens and giving rise to an estrogen-depleted environment, exhibit improved efficacy over tamoxifen in the adjuvant therapy of estrogen receptor (ER) positive breast cancer in postmenopausal women (7). Clinical trials evaluating these AIs showed a reduced incidence of contralateral primary breast cancer in the AI groups compared with tamoxifen (1 6); hence, AIs are currently being evaluated as chemopreventives in ongoing studies (8). AIs also exhibit reduced overall toxicity compared with tamoxifen (1 6, 9), but the toxicity profiles are different: tamoxifen is associated with increased incidences of thromboembolic events and endometrial cancer, whereas AIs are associated with decreased bone mineral density (BMD), coupled with an increased risk of bone fractures (10 12) and severe musculoskeletal pain that limits patient compliance (13, 14). Because the available third-generation AIs all exhibit similar efficacies, the selection of a specific AI for long-term adjuvant therapy of breast cancer and as a chemopreventive in healthy women at high risk for breast cancer will likely be determined by safety and tolerability profiles.

AIs fall into two classes, steroidal as represented by exemestane, which acts as a suicide inhibitor of aromatase, and nonsteroidal including anastrozole and letrozole, which reversibly block aromatase activity (7). Possibly due to its steroid structure, exemestane may exhibit a unique pharmacology distinct from the nonsteroidal AIs. In two preclinical studies by Goss et al. (15, 16), exemestane was given to female ovariectomized rats, an animal model

of osteoporosis, and found to reduce bone resorption markers and increase BMD and bone strength, whereas lowering serum cholesterol and low-density lipoprotein levels compared with ovariectomized controls. One of these preclinical studies also evaluated the nonsteroidal AI letrozole, but in contrast, found no benefit of letrozole on bone or lipid profiles (16). In a clinical study investigating the effects of 2 years of exemestane on bone compared with placebo without prior tamoxifen therapy in patients with surgically resected breast cancer at low risk for recurrence, exemestane did not enhance BMD loss in lumbar spine and only modestly enhanced BMD loss in the femoral neck compared with the placebo group (17). Interestingly, in this study, exemestane promoted bone metabolism by increasing levels of both bone resorption and formation markers (17). However, a clear-cut advantage of exemestane versus the nonsteroidal AIs on bone safety has not been shown in humans, possibly because all other clinical studies compared the AI to tamoxifen (9, 12, 18) or the AI to placebo with prior tamoxifen therapy (10, 11). Drawing conclusions from these studies is difficult because tamoxifen preserves BMD, thereby protecting against fractures, and withdrawal of tamoxifen may have lasting effects on BMD (19).

Maintenance of BMD in women is a known estrogenic effect (20). However, androgen receptors (AR) are also expressed in multiple bone cell types (21, 22), and studies show that androgens maintain BMD in ovariectomized rats (23, 24) and in women (21, 25–27). In ovariectomized rats, physiologic concentrations of androstenedione, a weak androgen and a substrate of aromatase, reduced loss of bone, and the antiandrogen bicalutamide abrogated this effect (23), but anastrozole did not (23). Therefore, the protective effect of androstenedione on maintenance of BMD was androgen mediated and not due to aromatization of androstenedione to estrogen. Furthermore, the non-aromatizable androgen $5\alpha$-dihydrotestosterone has been shown to stimulate bone growth in osteopenic ovariectomized rats (24). In pre- and postmenopausal women, endogenous androgen levels correlate with BMD (25, 26). Furthermore, a study comparing estrogen to a synthetic androgen in postmenopausal osteoporotic women showed that both steroids were equally effective in reducing bone resorption (27). Also, a 2-year double-blind trial showed that estrogen plus a non-aromatizable androgen significantly improved BMD over estrogen alone in surgically menopausal women (28). Therefore, exogenous androgens promote BMD maintenance in women when used alone (27) and in conjunction with estrogen (28).

Although exemestane does not bind ER, it is structurally related to androstenedione and has weak affinity for AR (29, 30). At high doses, exemestane exerts possible androgenic activity *in vivo* by inducing an increase in ventral prostate weight in immature castrated rats (29). Recently, Miki et al. (22) showed in human osteoblast hFOB and osteosarcoma Saos-2 cells that exemestane promoted proliferation, which was partially blocked by the antiandrogen hydroxyflutamide, and increased alkaline phosphatase activity. However, metabolites of exemestane may be mediating these effects. Exemestane is given p.o. at 25 mg/day and rapidly absorbed, showing peak plasma levels within 2 to 4 h and a direct relationship between dosage and peak plasma levels after single (10–200 mg) or repeated doses (0.5–50 mg; refs. 30, 31). Single-dose studies suggested that exemestane has a short elimination half-life, but multiple-dose studies show its terminal half-life to be about 24 h. Exemestane undergoes complex metabolism, and the primary metabolite in plasma has been identified as 17-hydroexemestane, which accumulates to a concentration of about 10% of its parent compound (30). Taking the possible action of metabolites into consideration, Goss et al. (16) administered 17-hydroexemestane to ovariectomized rats and found that it produced the same bone-sparing effects and favorable changes in circulating lipid levels as exemestane. Also, Miki et al. (22) stated that 17-hydroexemestane promoted proliferation of the osteoblast and osteosarcoma cells similar to exemestane, but the data were not shown, and the authors did not further explore 17-hydroexemestane activities. Additionally, Miki et al. (22) showed that the osteoblasts efficiently metabolized androstenedione to testosterone, which involves the reduction of the 17-keto group of androstenedione to a hydroxyl group. Similar metabolism would convert exemestane to 17-hydroexemestane, and thus, activities of exemestane in the osteoblasts may have been mediated by a metabolite of exemestane. Hence, a thorough investigation of exemestane and 17-hydroexemestane activities through ER and AR is warranted to provide evidence regarding whether exemestane could display a more favorable safety and toxicity profile than nonsteroidal AIs for long-term adjuvant use and as a chemopreventive of breast cancer in postmenopausal women. Therefore, we evaluated the pharmacologic actions of exemestane and its primary metabolite 17-hydroexemestane on ER- and AR-regulated activities in a range of cellular and molecular assays. First, we determined the relative binding affinity (RBA) of 17-hydroexemestane to ER$\alpha$ and AR. Next, using MCF-7 and T47D breast cancer cells, we examined the ability of 17-hydroexemestane to stimulate cell proliferation and cell cycle progression (Supplementary Material)[4] via ER and AR, to regulate ER- and AR-dependent transcription, and to modulate ER$\alpha$ and AR protein levels. Lastly, we investigated intermolecular interactions between 17-hydroexemestane and ER$\alpha$ and AR using molecular modeling.

## Materials and Methods

### Compounds and Cell Lines

Exemestane and 17-hydroexemestane were provided by Pfizer. Fulvestrant (ICI 182,780, Faslodex) and bicalutamide (Casodex) were provided by Dr. Alan E. Wakeling and Dr. Barrington J.A. Furr (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom), respectively. All other

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[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

compounds were obtained from Sigma-Aldrich, and cell culture reagents were from Invitrogen. All test agents were dissolved in ethanol and added to the medium at 1:1,000 (v/v). MCF-7/WS8 and T47D:A18 human mammary carcinoma cells, clonally selected from their parental counterparts for sensitivity to growth stimulation by $E_2$ (32), were used in all experiments indicating MCF-7 and T47D cells. Cells were maintained in steroid-replete RPMI 1640, but 3 days before all experiments, were cultured in steroid-free media as previously described (32, 33).

### Competitive Hormone-Binding Assays

Competitive hormone-binding assays were conducted using fluorescence polarization based ERα and AR Competitor Assay kits (Invitrogen) as previously described (34).

### Cellular Proliferation Assays

Cellular proliferation following 7 days in culture was determined by DNA mass per well in 12-well plates using the fluorescent DNA dye Hoechst 33258 as previously described (32).

### Reporter Gene Assays

Reporter gene assays were conducted by transfecting cells with either an ERE(5x)-regulated (pERE(5x)TA-ffLuc; ref. 33) or ARE(5x)-regulated (pAR-Luc; Panomics) firefly luciferase expression plasmid and co-transfected with a basal TATA promoter-regulated (pTA-srLuc) *Renilla* luciferase expression plasmid as previously described (33).

### Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was used to determine AR and ribosomal large phosphoprotein subunit P0 (RLP0; 36B4) mRNA levels as previously described (35).

### Immunoblot Analyses

Immunoblots, prepared as previously described (33), were probed with primary antibodies against AR (AR 441; Lab Vision), ERα (AER 611; Lab Vision), and β-actin (AC-15; Sigma-Aldrich).

### Molecular Modeling and Virtual Docking Calculations

The three-dimensional conformations for $E_2$, 17-hydroexemestane, exemestane, R1881, and dexamethasone were generated with Omega version 2.1 software (OpenEye Scientific Software). These compounds were docked using the following X-ray crystallographic structures: 1GWR (ERα co-complexed with $E_2$, 2.4-Å resolution; ref. 36) and 1XQ3 (AR co-complexed with R1881, 2.25-Å resolution; ref. 37). ERα and AR ligand-binding pockets were built using a ligand-centered box and the receptor-bound conformation of the respective ligand: $E_2$ (for 1GWR) and R1881 (for 1XQ3). The volume of the cavity differs for the two receptors: 648 Å$^3$ for 1GWR and 532 Å$^3$ for 1XQ3. All receptor and ligand bonds were kept rigid. The receptor structures were filled with water because ERα (38) and AR crystal structures (39) indicate that specific stable hydrogen bond (H-bond) networks form among particular water molecules, ligands, and amino acid side chains. Docking was done with FRED version 2.2 software (Open-Eye) using a short refinement step for the ligands within the receptor and using the MMFF94 force field. The best 30 conformations for each compound were compared and ranked by FRED's Chemscore function. For each ligand-docked receptor evaluated, the docked conformation with the lowest total intermolecular interaction energy (kJ/mol) was selected. To address whether water could be displaced by a compound during the process of binding, docking calculations were also done using receptors modeled with water removed as presented in Supplementary Table S1[4] and the differences between the methods in Supplementary Table S2.[4]

### Curve Fitting and Statistical Analyses

All statistical tests, curve fitting, and determination of half-maximal inhibitory concentrations ($IC_{50}$) and half-maximal effective concentrations ($EC_{50}$) were done using GraphPad Prism 4.03 (GraphPad Software). Significant differences were determined using one-way ANOVA with Bonferroni multiple comparison post-test.

## Results

### Experimentally Determined Binding of 17-Hydroexemestane and Exemestane to ERα and AR

Structures of the compounds relevant to these studies, the steroidal AI parent compound exemestane, its primary metabolite 17-hydroexemestane, $E_2$, and the synthetic non-aromatizable androgen R1881, are shown in Fig. 1A. Importantly, the only difference between parental exemestane and its metabolite 17-hydroexemestane is a hydroxyl group in the metabolite in place of a ketone in the parent compound at the 17β position, whereas both compounds share a 3-keto group. For steroidal estrogens, elimination or modification of the 17β-OH group reduces binding to ERα, but that of the 3-OH group is much more dramatic (40). For steroidal androgens, the trend is reversed; elimination or modification of the 17β-OH group is more significant for AR binding than that of the 3-keto group (41). The 3-keto group found in both exemestane and 17-hydroexemestane also favors binding to AR (41).

We tested the binding of exemestane and 17-hydroexemestane to ERα and AR using fluorescence polarization based competitive hormone-binding assays (Fig. 1B and C; Table 1). For purposes of comparison, compound affinities were arbitrarily categorized with respect to their RBAs as strong (100 to ≥1), moderate (<1 to ≥0.1), weak (<0.1 to ≥0.01), very weak (<0.01 to detectable binding defined as 50% competition), and inactive (compound did not compete for at least 50% binding). $E_2$ competitively bound ERα with an $IC_{50}$ of $1.33 \times 10^{-9}$ mol/L (RBA = 100; Fig. 1B), and R1881 competitively bound AR with an $IC_{50}$ of $1.34 \times 10^{-8}$ mol/L (RBA = 100; Fig. 1C). Considering ERα (Fig. 1B), both R1881 and 17-hydroexemestane competed for binding to ERα with $IC_{50}$s of $1.02 \times 10^{-6}$ mol/L (RBA = 0.130) and $2.12 \times 10^{-5}$ mol/L (RBA = 0.006), respectively, which categorized R1881 as a moderate and 17-hydroexemestane as a very weak ERα ligand. Neither exemestane nor dexamethasone significantly competed for binding to ERα. Regarding AR (Fig. 1C), 17-hydroexemestane and exemestane competed for binding to AR with $IC_{50}$s of $3.96 \times 10^{-8}$ mol/L (RBA = 33.8) and $2.03 \times 10^{-6}$ mol/L (RBA = 0.658), respectively, which classified
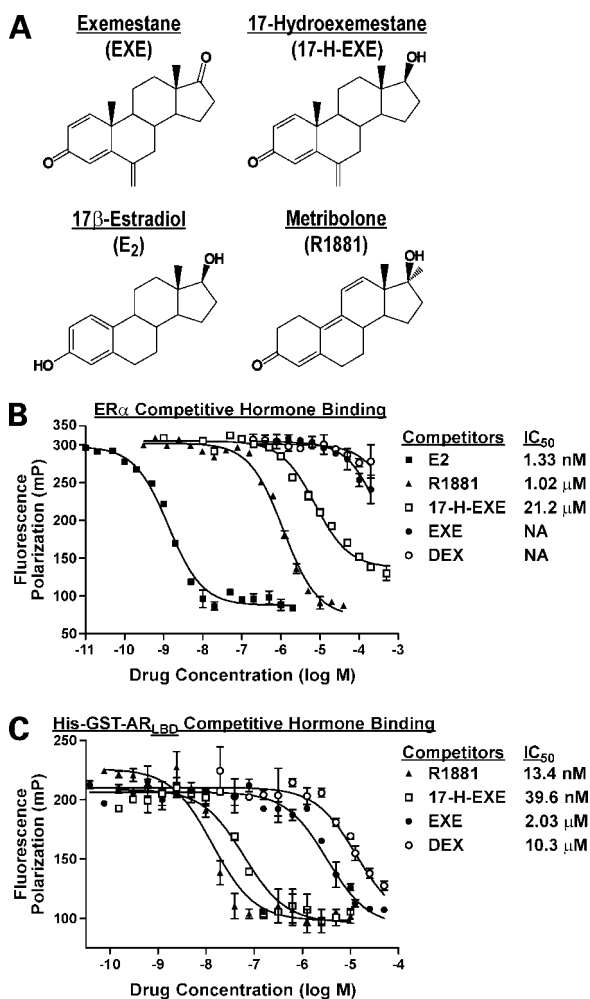
**Figure 1.** Compounds examined in this study and their RBAs for ERα and AR. **A,** structures of exemestane, its primary metabolite 17 hydroexemestane $E_2$, and R1881. ERα (**B**) and AR (**C**) fluorescence polarization based competitive hormone binding assays. Baculovirus produced human ERα and rat AR ligand binding domain tagged with a His glutathione *S* transferase epitope (His GST AR$_{LBD}$) were used at final concentrations of 15 and 25 nmol/L, respectively. The fluorescently labeled ERα and AR ligands, Fluormone ES2 and Fluormone AL Green, respectively, were both used at a final concentration of 1 nmol/L. The competing test compounds were $E_2$, R1881, 17 hydroexemestane, exemestane, and dexamethasone (DEX) as indicated. *Point,* mean of triplicate determinations; *bars,* 95% confidence intervals. Curve fitting was done using GraphPad Prism software (version 4.03). IC$_{50}$s corresponding to a half maximal shift in polarization values of the test compounds were determined using the maximum and minimum polarization values of the $E_2$ competitive binding curve for ERα or of the R1881 competitive binding curve for AR as appropriate.

17-hydroexemestane as a strong and exemestane as a weak AR ligand. However, dexamethasone would also be categorized as a weak AR ligand. Hence, the observed very weak ERα binding and strong AR binding of 17-hydroexemestane was consistent with what previously reported structure-activity relationships (40, 41) would have predicted due to reduction of the 17-keto group in exemestane to a 17β-OH in the metabolite.

## Proliferation Responses to 17-Hydroexemestane and Exemestane

We examined the effects of exemestane and 17-hydroexemestane on 7 days of proliferation in ERα- and AR-positive MCF-7 and T47D mammary carcinoma cells (Fig. 2). As expected, both cell lines were growth stimulated by $E_2$, with growth EC$_{50}$s of $1.7 \times 10^{-12}$ mol/L $E_2$ for MCF-7 cells (Fig. 2A) and $7.1 \times 10^{-12}$ mol/L $E_2$ for T47D cells (Fig. 2B). These growth responses to $E_2$ were completely blocked by fulvestrant (all *P* values <0.001), validating the $E_2$ responsiveness via ER in these cell lines.

Both cell lines were also growth stimulated by R1881 (Fig. 2A and B) and 17-hydroexemestane (Fig. 2C and D), whereas exemestane did not exert any significant effect on proliferation (Fig. 2C and D). Considering MCF-7 cells, R1881 exhibited a growth EC$_{50}$ of $2.4 \times 10^{-8}$ mol/L (Fig. 2A), or approximately 4 orders of magnitude higher than that of $E_2$. Similarly, 17-hydroexemestane exhibited a growth EC$_{50}$ of $2.7 \times 10^{-6}$ mol/L in MCF-7 cells (Fig. 2C) or approximately 6 orders of magnitude higher than that of $E_2$. These growth responses to R1881 and 17-hydroexemestane in MCF-7 cells were completely blocked by cotreatment with fulvestrant (Fig. 2A and B; both *P* values <0.001). Therefore, whereas R1881, a non-aromatizable synthetic androgen, stimulated growth of MCF-7 cells, it did so by acting through ER. Hence, at high concentrations, R1881 exerted estrogenic activity. Similarly, at high concentrations, 17-hydroexemestane also exerted estrogenic activity and stimulated growth of MCF-7 cells by acting through ER.

Interestingly, in T47D cells, the growth response to R1881 and 17-hydroexemestane followed an apparent bimodal pattern, which was different than in MCF-7 cells. In T47D cells, proliferative effects of high concentrations of R1881 ($5 \times 10^{-6}$ mol/L; Fig. 2B) and 17-hydroexemestane ($5 \times 10^{-6}$ mol/L; Fig. 2D) were only partially blocked by fulvestrant (both *P* values <0.001), down to the level of growth observed at nanomolar concentrations of these compounds. However, proliferative effects of lower concentrations of R1881 ($10^{-9}$ mol/L) and 17-hydroexemestane ($10^{-8}$ mol/L) were completely blocked by the antiandrogen bicalutamide (both *P* values <0.001). Based on these observed levels of inhibition by bicalutamide and fulvestrant, maximal concentrations at which R1881 and 17-hydroexemestane stimulated growth through AR-dependent activities were $10^{-7}$ and $10^{-6}$ mol/L, respectively, and above these concentrations, R1881 and 17-hydroexemestane stimulated growth through ER-dependent activities. Using this information to define concentration ranges in which these compounds exert AR-mediated or ER-mediated effects in T47D cells, the growth EC$_{50}$s via AR of R1881 and 17-hydroexemestane were $1.0 \times 10^{-10}$ mol/L (Fig. 2B) and $4.3 \times 10^{-10}$ mol/L (Fig. 2D), respectively. Similarly, the growth EC$_{50}$s via ER of R1881 and 17-hydroexemestane in T47D cells were $3.1 \times 10^{-7}$ mol/L (Fig. 2B) and $1.5 \times 10^{-6}$ mol/L (Fig. 2D), respectively. Hence, in T47D cells, both R1881 and 17-hydroexemestane stimulated growth via AR at lower

concentrations and via ER at higher concentrations. These results were consistent with the observed binding affinities of these compounds to ER$\alpha$ (Fig. 1B) and AR (Fig. 1C).

### Cell Cycle Progression Responses to 17-Hydroexemestane

As shown in Supplementary Fig. S1,[4] 17-hydroexemestane at $10^{-8}$ mol/L acted through AR to stimulate S-phase entry in T47D cells by 1.9-fold ($P < 0.001$) but, at $5 \times 10^{-6}$ mol/L, acted through ER to stimulate S-phase entry in MCF-7 cells by 2.2-fold ($P < 0.001$). Hence, 17-hydroexemestane effects on cell cycle progression were consistent with its effects on proliferation (Fig. 2).

### Regulation of ER$\alpha$ and AR Transcriptional Activities by 17-Hydroexemestane

Next, we investigated the ability of 17-hydroexemestane to regulate ER and AR transcriptional activity by transfecting cells with an ERE(5x)-regulated or ARE(5x)-regulated dual-luciferase plasmid set, treating cells with test compounds, and measuring dual-luciferase activity 44 h after treatment (Fig. 3A C). $E_2$ at $10^{-10}$ mol/L induced ERE(5x)-regulated transcription by 19.4-fold in MCF-7 cells (Fig. 3A; $P < 0.001$), and 11.3-fold in T47D cells (Fig. 3B; $P < 0.001$) compared with control-treated cells; this $E_2$-induced transcriptional activity was blocked by fulvestrant (both $P$ values <0.001), validating dependence on ER for ERE(5x)-regulated transcription. At high sub-micromolar and micromolar concentrations, R1881 stimulated ERE(5x)-regulated transcription in both cell lines, with maximal inductions of 22.7-fold at $5 \times 10^{-6}$ mol/L in MCF-7 cells (Fig. 3A; $P < 0.001$), and 7.9-fold at $5 \times 10^{-6}$ mol/L in T47D cells (Fig. 3B; $P < 0.001$) compared with control-treated cells. The ability of R1881 at $5 \times 10^{-6}$ mol/L to induce ERE(5x)-regulated transcription was blocked by fulvestrant (Fig. 3A and B; both $P$ values <0.001), indicating that at high concentrations, R1881 acted as an estrogen. In a similar manner as R1881, 17-hydroexemestane stimulated ERE(5x)-regulated transcription in a concentration-dependent manner at sub-micromolar and micromolar concentrations

(Fig. 3A and B). At $5 \times 10^{-6}$ mol/L, 17-hydroexemestane maximally induced ERE(5x)-regulated transcription by 7.7-fold in MCF-7 cells (Fig. 3A; $P < 0.001$) and 3.3-fold in T47D cells (Fig. 3B; $P < 0.001$) compared with control-treated cells; this transcriptional activation was blocked by fulvestrant (both $P$ values <0.001). Therefore, at high concentrations, 17-hydroexemestane acted as an estrogen and induced ER transcriptional activity.

In a similar manner, AR-dependent transcriptional activity was investigated. T47D cells showed a concentration-dependent induction of ARE(5x)-regulated transcription in response to R1881, with $10^{-9}$ mol/L R1881 inducing transcription by 8.5-fold and $10^{-6}$ mol/L R1881 maximally inducing transcription by 12.7-fold relative to control-treated cells (Fig. 3C; both $P$ values <0.001). Bicalutamide blocked $10^{-9}$ mol/L R1881-mediated induction of ARE(5x)-regulated transcription (Fig. 3C; $P < 0.001$), confirming dependence on AR. MCF-7 cells failed to respond to $10^{-6}$ mol/L R1881 with induction of ARE(5x)-regulated transcription (data not shown), although these cells express AR protein. This supports our prior results that T47D cells were growth stimulated by R1881 through an AR-dependent mechanism (Fig. 2B), but that MCF-7 cells were not (Fig. 2A). As expected, $10^{-6}$ mol/L $E_2$ failed to induce ARE(5x)-regulated transcription (Fig. 3C). Next, 17-hydroexemestane was evaluated in T47D cells and, in a concentration-dependent manner, induced ARE(5x)-regulated transcription with maximal induction of 4.7-fold occurring at $5 \times 10^{-6}$ mol/L relative to control treatment (Fig. 3C; $P < 0.001$). However, because high concentrations of 17-hydroexemestane were needed to induce this synthetic ARE(5x)-regulated promoter, we tested whether lower concentrations of 17-hydroexemestane could modulate endogenous AR mRNA expression, which is known to be negatively feedback regulated by its gene product (42). Using real-time PCR, AR mRNA levels were determined in T47D cells following 24 h of treatment with test compounds (Fig. 3D). R1881 at $10^{-9}$ mol/L significantly down-regulated

**Table 1.** Compound affinity for ER$\alpha$ and AR determined experimentally using a competitive hormone binding assay (Fig. 1B and C), and by computer docking in which receptors were modeled as filled with water

| Compound | Receptor | Competitive hormone binding | | | Intermolecular interaction energy (kJ/mol) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | IC$_{50}$ (mol/L) | 95% CI (mol/L) | RBA (%) | Total score | Lipophilic | H bond | Steric clash | RTB penalty |
| $E_2$ | ER$\alpha$ | $1.33 \times 10^{-9}$ | 1.18 $1.49 \times 10^{-9}$ | 100 | −31.90 | −25.96 | −6.00 | 0.06 | 0 |
| R1881 | ER$\alpha$ | $1.02 \times 10^{-6}$ | 0.90 $1.15 \times 10^{-6}$ | 0.130 | −29.96 | −26.01 | −4.32 | 0.37 | 0 |
| 17 Hydroexemestane | ER$\alpha$ | $2.12 \times 10^{-5}$ | 1.73 $2.61 \times 10^{-5}$ | 0.006 | −29.14 | −27.73 | −3.34 | 1.93 | 0 |
| Exemestane | ER$\alpha$ | NA | | | −27.33 | −25.98 | −3.34 | 1.99 | 0 |
| Dexamethasone | ER$\alpha$ | NA | | | −23.71 | −29.70 | −4.18 | 9.07 | 1.10 |
| R1881 | AR | $1.34 \times 10^{-8}$ | 1.00 $1.79 \times 10^{-8}$ | 100 | −32.75 | −28.47 | −4.56 | 0.28 | 0 |
| 17 Hydroexemestane | AR | $3.96 \times 10^{-8}$ | 2.74 $5.71 \times 10^{-8}$ | 33.8 | −31.95 | −30.54 | −4.76 | 3.35 | 0 |
| Exemestane | AR | $2.03 \times 10^{-6}$ | 1.39 $2.97 \times 10^{-6}$ | 0.658 | −26.48 | −28.80 | −2.11 | 4.43 | 0 |
| Dexamethasone | AR | $1.03 \times 10^{-5}$ | 0.75 $1.43 \times 10^{-5}$ | 0.130 | −24.53 | −32.21 | −2.49 | 9.07 | 1.10 |

Abbreviations: RTB Penalty, rotable bond penalty; NA, not applicable; test compound did not compete for at least 50% binding of ER$\alpha$.
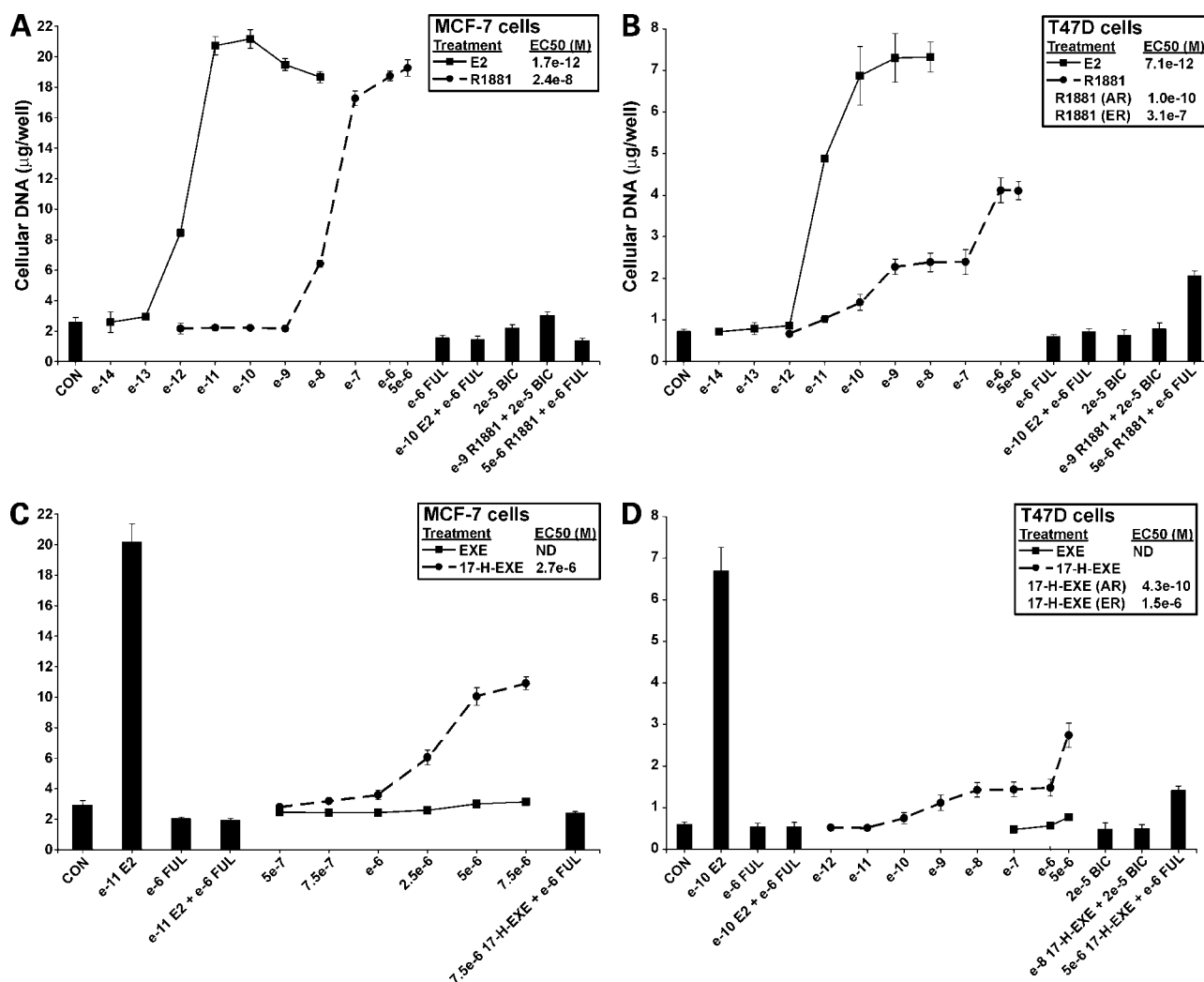
**Figure 2.** 17 Hydroexemestane and R1881 stimulate cellular proliferation. DNA based cellular proliferation assays of (**A**) MCF 7 cells treated with $E_2$ and R1881, (**B**) T47D cells treated with $E_2$ and R1881, (**C**) MCF 7 cells treated with exemestane and 17 hydroexemestane, and (**D**) T47D cells treated with exemestane and 17 hydroexemestane. Cells were cultured in steroid free medium for 3 d before the assays. MCF 7 cells were seeded at 15,000 cells per well and T47D cells at 20,000 cells per well in 12 well plates. Cells were treated on days 0 (the day after seeding), 3, and 6, and then collected on day 7. Cellular DNA quantities were determined using the fluorescent DNA binding dye Hoechst 33258 and compared against a standard curve. Data shown represent the mean of four replicates and SDs. DNA values were fitted to a sigmoidal dose response curve and growth $EC_{50}$s calculated using GraphPad Prism 4.03 software. At high concentrations, 17 hydroexemestane and R1881 increased growth via ER in both cell lines but, at low concentrations, stimulated growth via AR selectively in T47D cells. Abbreviations: CON, control; FUL, fulvetsrant; BIC, bicalutamide.

AR mRNA expression by 48% ($P < 0.001$), whereas $10^{-9}$ mol/L $E_2$ did not (Fig. 3D). Bicalutamide prevented R1881-mediated decrease in AR mRNA expression (Fig. 3D), validating that AR mRNA levels were negatively feedback regulated. Similarly, a low $10^{-8}$ mol/L concentration of 17-hydroexemestane led to a 41% decrease in AR mRNA levels ($P < 0.01$), with increased 17-hydroexemestane concentrations further decreasing AR mRNA expression (Fig. 3D). Bicalutamide blocked 17-hydroexemestane mediated down-regulation of AR mRNA expression ($P < 0.01$), whereas fulvestrant did not (Fig. 3D). Therefore, 17-hydroexemestane acted as an androgen via AR to feedback-regulate the expression of endogenous AR mRNA in T47D cells.

## Modulation of AR and ERα Protein Levels by 17-Hydroexemestane

Androgens and estrogens modulate protein expression levels of their cognate receptors. R1881 stabilizes AR protein allowing its accumulation (43), whereas $E_2$ promotes ERα degradation in a cell type dependent manner (32). Therefore, we investigated the effects of 17-hydroexemestane on AR and ERα protein levels by treating cells with test compounds for 24 h and analyzing receptor levels by immunoblotting. $E_2$ decreased ERα protein levels in MCF-7 (Fig. 4A), but not T47D cells (Fig. 4B), as we have previously shown (32). As expected, fulvestrant promoted ERα protein degradation in both cell lines. $E_2$ did not significantly affect AR protein accumulation in MCF-7 cells

(Fig. 4A), but did down-regulate AR protein levels in T47D cells (Fig. 4B). Also, fulvestrant and $E_2$ plus fulvestrant treatments did not significantly affect AR protein levels in MCF-7 cells (Fig. 4A), but did modestly up-regulate AR protein levels in T47D cells (Fig. 4B). As expected, R1881 caused an increase in accumulation of AR protein in both cell lines (Fig. 4A and B), likely by stabilizing the protein (43). Next, we characterized the effects of low $10^{-8}$ mol/L and high $5 \times 10^{-6}$ mol/L concentrations of 17-hydro-exemestane on ER$\alpha$ and AR expression. The high $5 \times 10^{-6}$ mol/L concentration of 17-hydroexemestane led to decreased ER$\alpha$ protein levels in MCF-7 (Fig. 4A), but not in T47D cells (Fig. 4B); this pattern indicates that $5 \times 10^{-6}$ mol/L

17-hydroexemestane acted as an estrogen to regulate ER$\alpha$ protein in a cell type dependent manner. Similar to R1881, treatment with low $10^{-8}$ mol/L or high $5 \times 10^{-6}$ mol/L concentrations of 17-hydroexemestane led to increased AR protein accumulation in both cell lines (Fig. 4A and B), indicating that 17-hydroexemestane acted as an androgen likely by stabilizing AR protein. Therefore, 17-hydroexemestane modulated ER$\alpha$ and AR protein accumulation as would an estrogen and an androgen, respectively.

## Molecular Docking of 17-Hydroexemestane and Exemestane to ER$\alpha$ and AR

To investigate the mechanism by which 17-hydroexemestane binds ER$\alpha$ as a very weak ligand and AR as a
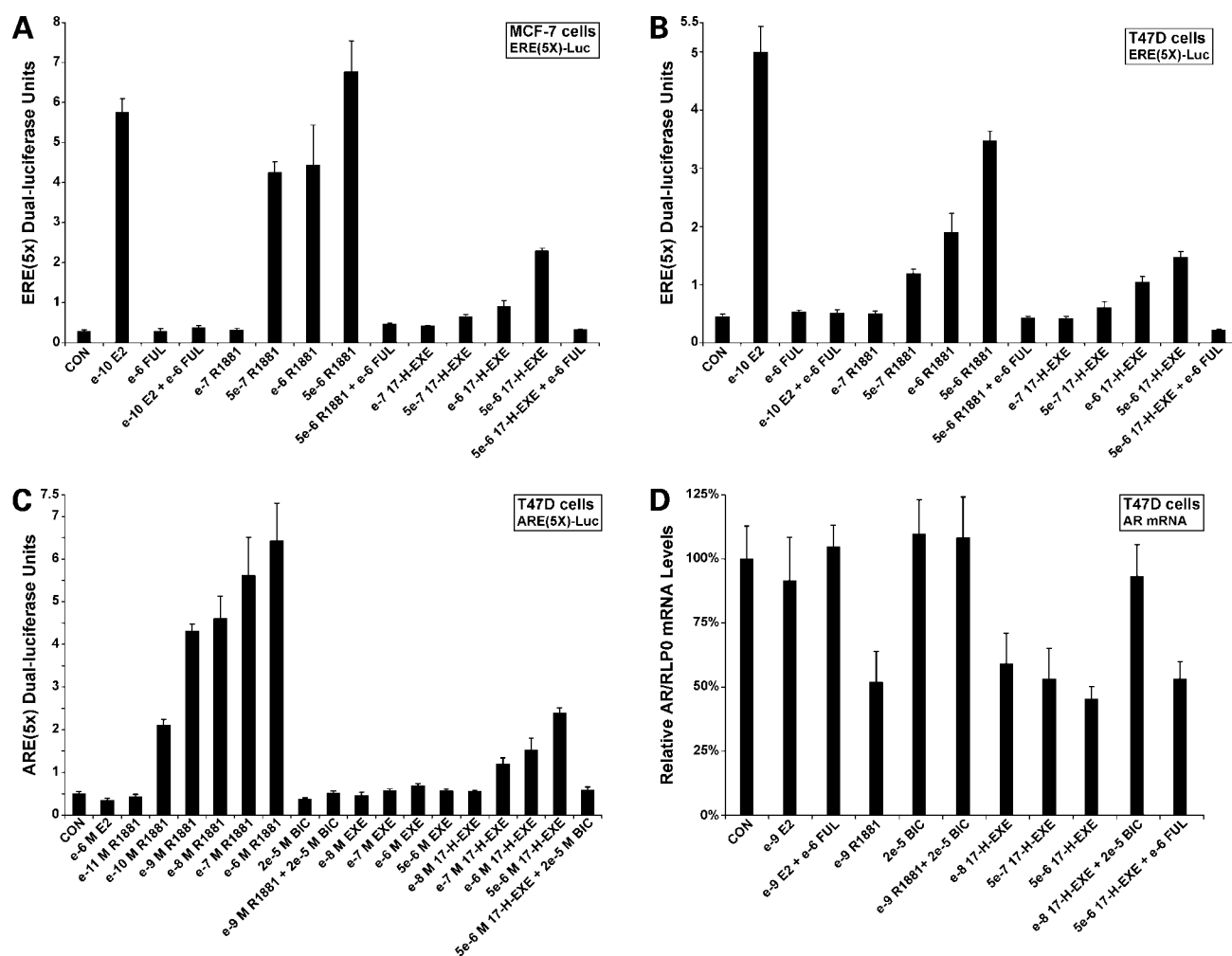


**Figure 3.** 17 Hydroexemestane and R1881 regulate ER transcriptional activity at high concentrations and AR transcriptional activity at low concentrations. ERE(5x) regulated dual luciferase activity in (**A**) MCF 7 cells and (**B**) T47D cells. (**C**) ARE(5x) regulated reporter gene activity in T47D cells. *A  C*, Under steroid free conditions, cells were transiently transfected with pERE(5x)TA ffLuc or pARE(5x) Luc (firefly luciferase reporter plasmids) and the internal normalization control pTA srLuc (*Renilla* luciferase reporter plasmid). Four hours after transfection, cells were treated as indicated and then again the following day. Cells were assayed 44 h after transfection for dual luciferase activity. Data shown are the mean of triplicate determinations and associated SDs. 17 Hydroexemestane and R881 stimulated ERE(5x) regulated transcription in MCF 7 and T47D cells and ARE(5x) regulated transcriptional activity in T47D cells. **D,** AR mRNA levels in T47D cells as determined by real time PCR. T47D cells were treated as indicated for 24 h. RNA was isolated and converted to cDNA. Continuous accumulation of PCR products was monitored using the double strand specific DNA dye SYBR Green. Quantitative measurements of AR mRNA and the endogenous normalization control RLP0 mRNA were determined by comparison to a standard curve of known quantities of serially diluted AR or RLP0 PCR product. The data represent the mean and SDs of three independent samples, each of which was measured in triplicate. 17 Hydroexemestane and R881 down regulated AR mRNA levels at nanomolar concentrations in an AR dependent manner.
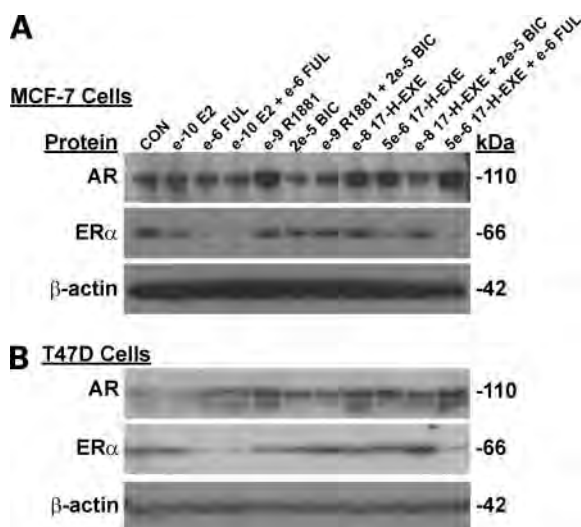
**Figure 4.** 17 Hydroexemestane modulates AR and ERα protein levels. Immunoblot analysis of AR and ERα in (**A**) MCF 7 cells and (**B**) T47D cells. Cells were treated as indicated for 24 h, and 20 μg of cellular protein were resolved by 4% to 12% SDS PAGE and then transferred to a nylon membrane. Membranes were probed for AR, ERα, and β actin, and immunoreactive bands were visualized by chemiluminescence and autoradiography. Cropped blots are shown. 17 hydroexemestane up regulated AR protein levels at $10^{-8}$ mol/L in both cell lines and down regulated ERα in MCF 7 cells at $5 \times 10^{-6}$ mol/L.

strong ligand, molecular models were constructed *in silico*. The trends in the computed intermolecular interaction energies matched the experimentally determined RBAs (Table 1). Superimposition of the docked and crystallographic structures of $E_2$ complexed with ERα (Fig. 5A) and of R1881 complexed with AR (Fig. 5B) showed that the docking models recapitulated the molecular recognition patterns of the crystal structures.

Considering ERα, the intermolecular interaction energies of R1881 and 17-hydroexemestane were less favorable than $E_2$ by 1.94 and 2.76 kJ/mol, respectively, due to decreased H-bond interactions and increased steric clash (Table 1). Exemestane was much less favorable than $E_2$ by 4.57 kJ/mol (Table 1). Hence, the 17β-OH group of 17-hydroexemestane compared with the 17-keto group of exemestane contributed −1.81 kJ/mol toward increased affinity for ERα. Interestingly, the docking calculations suggested that the higher affinity of 17-hydroexemestane over exemestane for ERα was not due to increased H-bonding mediated by the 17β-OH group, but rather increased lipophilic interactions (Table 1) due to a slight repositioning of the compound as a consequence of 17β-OH group. In the $E_2$ docked to ERα model, H-bonds between $E_2$ and Glu[353], Arg[394], and His[524] side chains were observed (Fig. 5A). In the docked 17-hydroexemestane to ERα model (Fig. 5C), the same Arg[394] and His[524] interactions were maintained, except that there was a loss of the Glu[353] interaction. The R1881 docked to ERα model is shown in Supplementary Fig. S2A.[4]

Considering AR, the intermolecular interaction energy of 17-hydroexemestane was only 0.8 kJ/mol less favorable

than R1881, whereas exemestane was significantly less favorable than R1881 by 6.27 kJ/mol (Table 1). Docking of 17-hydroexemestane to AR, compared with the parent drug exemestane, indicated that 17-hydroexemestane exhibited improved lipophilic interactions by −2.11 kJ/mol, more favorable H-bonding interactions by −2.65 kJ/mol, and decreased steric clash by −1.08 kJ/mol. Hence, the 17β-OH group in 17-hydroexemestane compared with the 17-keto group in exemestane contributed −5.47 kJ/mol toward higher affinity for binding AR (Table 1). In the R1881 docked to AR model, H-bonds between R1881 and Asn[705], Gln[711] and Arg[752] were observed (Fig. 5B). The OH side chain of Thr[877] was in close proximity to both docked R1881 (Fig. 5B) and 17-hydroexemestane (Fig. 5D), but the angle was not favorable for H-bonding. Docking of 17-hydroexemestane to AR (Fig. 5D) indicated a short 2.78-Å H-bond between the 17β-OH group of the ligand and Asn[705], but not between the 3-keto group of the ligand and Gln[711] and Arg[752]. Hence, the short 2.78-Å H-bond observed in the 17-hydroexemestane docked to AR model was important in mediating high affinity binding. The exemestane docked to AR model is shown in Supplementary Fig. S2B.[4]

## Discussion

We observed that 17-hydroexemestane, the primary metabolite of exemestane, bound to ERα as a very weak ligand and acted through ER at high sub-micromolar and micromolar concentrations to stimulate growth, promote cell cycle progression, induce ERE-regulated reporter gene expression, and down-modulate ERα protein levels in breast cancer cells. However, we also observed that 17-hydroexemestane bound to AR as a strong ligand and found in T47D cells that 17-hydroexemestane stimulated growth, induced cell cycle progression, down-modulated AR mRNA expression, and stabilized AR protein levels, with all of these effects occurring at low nanomolar concentrations and blocked by bicalutamide. Moreover, computer docking indicated that the 17β-OH group of 17-hydroexemestane versus the 17-keto group of exemestane contributed significantly more toward increasing affinity to AR than to ERα. Molecular modeling also indicated that 17β-OH group of 17-hydroexemestane interacted with AR through an important H-bond of Asn[705], a conserved recognition motif employed by R1881. Therefore, we propose that the primary mechanism of action of exemestane *in vivo* is mediated by 17-hydroexemestane regulating AR activities.

The Food and Drug Administration label for exemestane (Aromasin; Pfizer) reports that in postmenopausal women with advanced breast cancer, the mean AUC (area under the curve) values of exemestane following repeated doses was 75.4 ng·h/mL (254 nmol·h/L), which was almost twice that in healthy postmenopausal women (41.4 ng·h/mL; 140 nmol·h/L; ref. 31). Because circulating levels of 17-hydroexemestane can reach about 1/10 the level of the parent compound (30), we hypothesize that circulating levels of 17-hydroexemestane are sufficient to bind AR and

regulate AR-dependent activities. Furthermore, a subpopulation of patients may exist who metabolize exemestane at higher rates, leading to correspondingly higher circulating 17-hydroexemestane levels. For instance, one of three patients administered 800 mg of exemestane, the highest dose evaluated, achieved 17-hydroexemestane plasma levels approximately one-half the level of the parent compound (30). Based on our results, we would predict that higher circulating levels of 17-hydroexemestane would associate with decreased rates of BMD loss and risk of bone fractures in postmenopausal women. We suggest that circulating levels of 17-hydroexemestane and exemestane should be determined in clinical trials and correlated to disease outcome and toxicity profiles such as BMD loss.

Although the clinical studies reported thus far were not designed to directly compare one AI versus another, comparisons in the rate of BMD loss from baseline to year 1, and from year 1 to 2 can be made. In the bone safety subprotocol of the IES (Intergroup Exemestane Study) trial,

the rate of BMD loss was greatest within 6 months of switching from tamoxifen to exemestane at −2.7% in the lumbar spine and −1.4% in the hip, but thereafter, BMD loss progressively slowed in months 6 to 12 and again in months 12 to 24 to only −1.0% and −0.8% in the lumbar spine and hip, respectively (10), which is in the same range as would be expected for postmenopausal women in general. However, in the bone safety substudy of the MA.17 trial, patients administered letrozole experienced a relatively constant rate of BMD loss for 2 years: at 12 months, the rate of BMD loss from baseline was −3.3% and −1.43% in lumbar spine and hip, respectively, and from year 1 to year 2, −2.05% and −2.17% in lumbar spine and hip, respectively (11). In the bone substudy of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial, the rate of BMD loss from baseline to year 1 was −2.2% in lumbar spine and −1.5% in hip and from year 1 to year 2, −1.8% in lumbar spine and −1.9% in hip (18). Collectively, these results suggest that after the initial
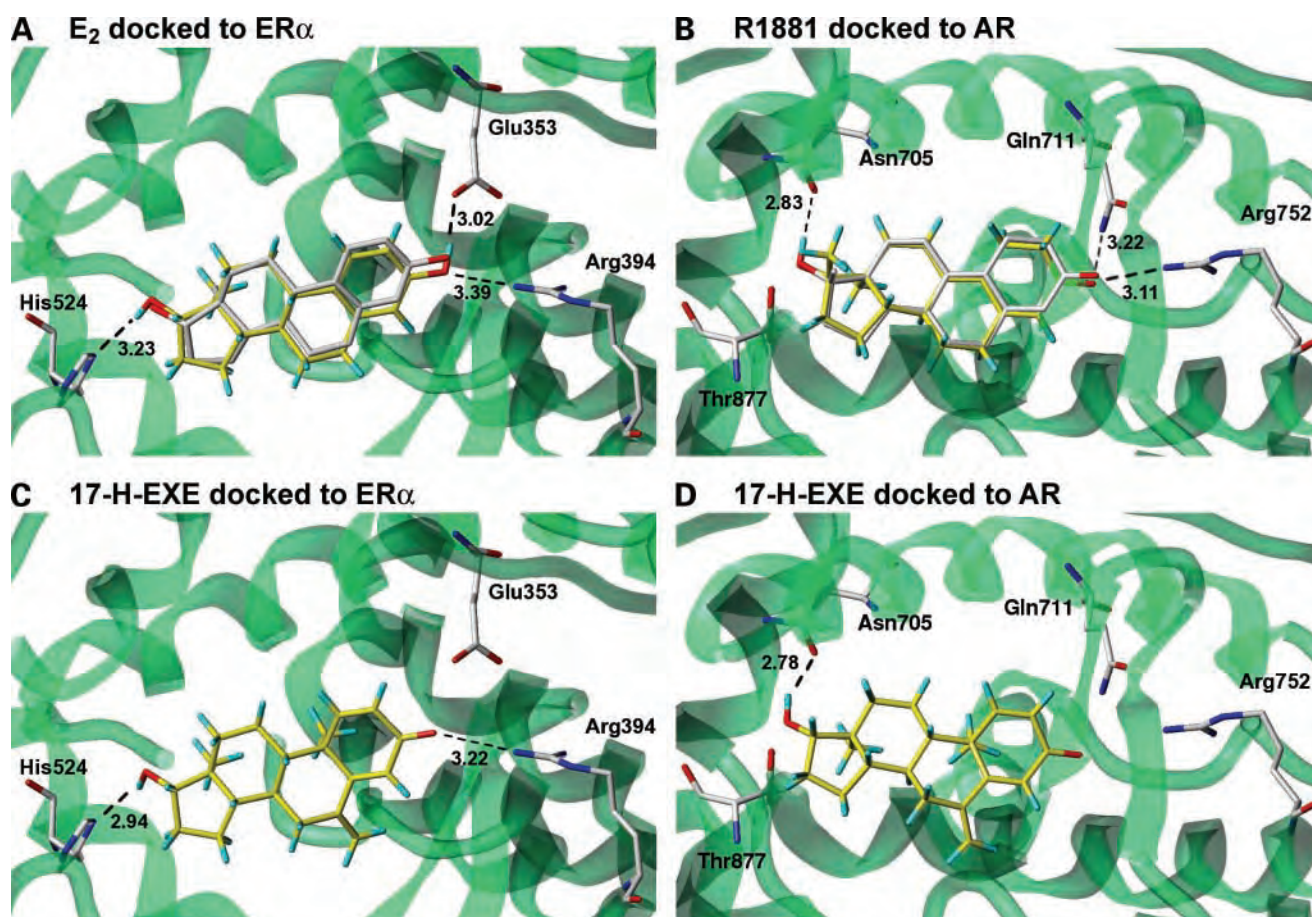


**Figure 5.** Intermolecular interactions of ligands complexed with ERα and AR by computer docking. **A,** superposition of $E_2$ from the X ray crystal structure (*gray*) and modeled $E_2$ (*yellow*) docked to ERα. **B,** superposition of R1881 from the crystal structure (*gray*) and modeled R1881 (*yellow*) docked to AR. **C,** modeled 17 hydroexemestane docked to ERα. **D,** modeled 17 hydroexemestane docked to AR. *Cyan, red,* and *blue,* hydrogen, oxygen, and nitrogen atoms, respectively. *Green,* carbon backbone of the protein. Hydrogens from the X ray crystal conformations of $E_2$ (**A**) and R1881 (**C**) were omitted. H bonds were shown to the modeled compound conformations only. *Dashed lines,* intermolecular H bonds up to 3.5 Å; their length in angstroms is indicated.

12 months of AI therapy, exemestane may be associated with slower rates of BMD loss compared with nonsteroidal AIs. Furthermore, although not directly comparable, the fracture rate per 1,000 woman-years in the ATAC trial was 22.6 for anastrozole and 15.6 for tamoxifen (1), whereas in the IES trial, the incidence rate per 1,000 woman-years for multiple fractures was 19.2 for exemestane and 15.1 for tamoxifen (10). These results show that although both anastrozole and exemestane were associated with higher fracture rates than tamoxifen, they also suggest that exemestane may be associated with a lower fracture rate than anastrozole. Clinical trials now under way to directly compare the different AIs will hopefully provide clear results.

Androgens regulate growth of normal and neoplastic mammary cells in a cell type-specific manner, either by inhibiting or stimulating growth (44). However, the mechanisms by which androgens via AR regulate breast cancer growth remain elusive. Female AR knock-out mice exhibit decreased ductal branching and terminal end buds in prepubertal animals and retarded lobuloalveolar development in adult animals (45). Likewise, targeted disruption of AR in MCF-7 cells also leads to severe inhibition of proliferation (45). Epidemiologic analyses indicate a positive correlation between androgen levels and the incidence of breast cancer; meta-analysis from nine prospective studies showed that a doubling in testosterone concentrations in postmenopausal women translated into an increased relative risk of 1.42 unadjusted and 1.32 adjusted for $E_2$ (46). AR status in breast cancer associates with both positive and negative indicators and clinical outcome. AR expression has been found in 84% (47) to 91% (48) of clinical breast cancers, and associated with ER status, but has also been found in 49% of ER-negative tumors (49). Patients with tumors that coexpress AR with ER and progesterone receptor have shown longer disease-free survival (DFS) than patients whose tumors were negative for all three receptors (48), but AR protein levels have also served as an independent predictor of axillary metastases in multivariate analysis (47) Furthermore, AR expression has correlated with decreased histopathologic grade, greater age, and postmenopausal status, but also lymph node positive status (50). In AR-positive/ER-negative tumors, AR expression again associated with positive and negative indicators/outcome such as increased age, postmenopausal status, and longer DFS but also tumor grade, tumor size, and HER-2/neu overexpression (49).

Patients who fail AI therapy, whether the AI was steroidal or nonsteroidal, likely harbor tumor cells that have been selected for growth in an estrogen-depleted environment and, hence, are not dependent on ER activity for survival. Not all androgens are metabolized by aromatase to estrogens; for instance, dihydrotestosterone cannot be converted to an estrogen by aromatase (44). Thus, a possible mechanism for failure of AI therapy in the clinic is androgen-stimulated breast cancer growth, a largely unrecognized alternative mechanism. We observed cellular proliferation of T47D cells in response to R1881 and 17-hydroexemestane, and these effects were blocked by

bicalutamide. Therefore, T47D cells contain a functional AR signaling pathway that promoted growth in the absence of estrogen. Because functional AR signaling could be etiologically involved in a subpopulation of clinical breast cancers, those patients who have AR-positive tumors and achieve high circulating levels of 17-hydroexemestane, yet whose disease progresses while on exemestane therapy, may respond to AR-based therapy such as the antiandrogen bicalutamide.
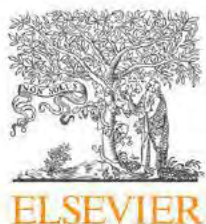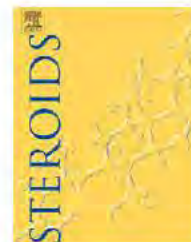
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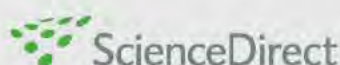
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## Review

# New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer

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ABSTRACT

The metabolism of tamoxifen is being redefined in the light of several important pharmacological observations. Recent studies have identified 4-hydroxy N-desmethyltamoxifen (endoxifen) as an important metabolite of tamoxifen necessary for antitumor actions. The metabolite is formed through the enzymatic product of CYP2D6 which also interacts with specific selective serotonin reuptake inhibitors (SSRIs) used to prevent the hot flashes observed in up to 45% of patients taking tamoxifen. Additionally, the finding that enzyme variants of CYP2D6 do not promote the metabolism of tamoxifen to endoxifen means that significant numbers of women might not receive optimal benefit from tamoxifen treatment. Clearly these are particularly important issues not only for breast cancer treatment but also for selecting premenopausal women, at high risk for breast cancer, as candidates for chemoprevention using tamoxifen.

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## Contents

# 1.    Introduction

The aim of the body's biotransformation mechanisms is to prevent potentially toxic xenobiotic substances that include drugs, from damaging the body. That being the case, an orally active medicine must overcome numerous challenges to reach a target organ and produce the appropriate pharmacological effect at a receptor system. There is not one but several stages of biotransformation of a lipophilic drug such as tamoxifen that are designed to enhance the hydrophilic nature of the chemical so it can be rapidly eliminated. The stages of biotransformation are called phases I, II and III.

Phase I metabolism enhances the water solubility of a lipophilic chemical by hydroxylating an aromatic compound to become a phenol or hydrolyzing an esterified compound. These reactions are conducted by the family of cytochrome $P_{450}$ enzymes referred to as CYP's. Phase II metabolism further increases the water solubility of the Phase I product by attaching highly water soluble entities. In the case of selective estrogen receptor modulators (SERMs) sugars (glucuronic acid) and salts (sulfates) are the most important conjugation products. In contrast, the phase III system is efflux pump molecules (also known as *p*-glycoproteins and multi-drug resistance transports protein) that exclude unmetabolized drugs from the epithelial cells of the intestinal tract immediately upon absorption.

In general terms, the ingested SERM must survive "first pass" metabolism from the intestine to the liver to have any chance of reaching target organs around the body. The general principles are illustrated in Fig. 1 where the SERM is biotransformed by CYPs in the intestinal wall and Phase II metabolism occurs via intestinal bacteria. A fraction of the administered dose is then absorbed into the hepatic portal vein and further biotransformed by phase I CYPs and/or glucuronidated or sulfated in phase II metabolism in the liver. By way of example, only 2% of the administered raloxifene survives and is bioavailable for systemic distribution [1].

# 2.    Tamoxifen, the first SERM

The nonsteroidal antiestrogen tamoxifen (ICI 46,474 Nolvadex®) is a pioneering medicine [2] used to treat all stages of breast cancer in more than 120 countries throughout the world. The compound ICI 46,474 was discovered in the Fertility Control Program at Imperial Chemical Industries (ICI Pharmaceuticals Division, now AstraZeneca) in Alderley Park, Cheshire, England in the early 1960s [3–5]. The drug was found to be an extremely potent postcoital contraceptive in the rat [4,5]. Unfortunately, ICI 46,474 did not exhibit antifertility properties in women, in fact, quite the opposite, it induced ovulation [6,7]. As a result, the medicine was, at one time, marketed in the United Kingdom for the induction of ovulation in subfertile women with a functional hypothalamo-pituitary-ovarian axis.

There is a known link between estrogen and the initiation and growth of some breast cancers [8] so the nonsteroidal antiestrogen ICI 46,474 was tested as a potential treatment for advanced breast cancer in postmenopausal women. The

antiestrogen produced response rates of 25–35% in unselected patients comparable to diethylstilbestrol and high dose androgen therapy, the standard endocrine therapies at the time [9,10]. However, fewer side effects were noted with tamoxifen [9,10]. As a result, the drug was approved as a palliative option for the hormonal treatment of breast cancer in the UK in 1973. There the story may have ended had not tamoxifen been reinvented as the first targeted therapy for breast cancer [2].

The seminal observations by Elwood Jensen that estrogen action is mediated by the estrogen receptor (ER) [11,12] in its target tissues (uterus, vagina, pituitary and breast tumors) opened the door to targeting tamoxifen to select patients with the ER in their metastatic tumor [13,14]. However, a strategic plan was developing to use tamoxifen in a broader range of patient populations. Laboratory studies conducted in the 1970s showed that tamoxifen blocked estrogen binding to the ER [15–17], should be used as a long-term adjuvant therapy to suppress tumor recurrence [18–20] and the drug also had potential as a chemopreventive agent [21,22].

Clinical studies subsequently confirmed that long-term adjuvant tamoxifen therapy, targeted to the patients with ER positive breast cancers, significantly decreased the death rate from the disease [23] and contributes to the current decline in death from breast cancer nationally [24]. Overall, the strategy of targeted long-term "antiestrogenic" [25] treatment for breast cancer has presaged the current fashion of targeting anticancer agents to other organ sites in the body.

Despite the fact that aromatase inhibitors show superiority over tamoxifen as adjuvant therapy in postmenopausal women [26–29], several issues have surfaced that have retained tamoxifen as a useful therapeutic agent worldwide. The medicine is extremely cheap compared to aromatase inhibitors so tamoxifen remains an essential anticancer agent in undeveloped countries or in countries with under-funded managed healthcare systems. Furthermore, tamoxifen is the only appropriate antiestrogenic therapy for premenopausal women whether they are being treated for breast cancer or whether chemoprevention is being considered [30]. For these reasons, new knowledge that can enhance the appropriate use of an established drug is of value to improve healthcare.

There are current initiatives to translate emerging knowledge on genetic variations in drug metabolism to target patient populations [31]. It is reasoned that by applying pharmacogenomic tests to specific patient populations, there will be fewer surprises with side effects, drug interactions, and a higher probability of increasing therapeutic effectiveness in the treatment or prevention of disease. The promise of practical progress is exemplified in this article using tamoxifen as the model drug.

Tamoxifen is a prodrug and can be metabolically activated to 4-hydroxytamoxifen [32–34] or alternatively can be metabolically routed via N-desmethyltamoxifen to 4-hydroxy-N-desmethyltamoxifen [35,36] (Fig. 2). The hydroxy metabolites of tamoxifen have a high binding affinity for the ER [32,37]. The finding that the enzyme produced by CYP2D6 activates tamoxifen to hydroxylated metabolites 4-hydroxytamoxifen and endoxifen [38] has implications for cancer therapeutics. Women with enzyme variants that cannot make endoxifen may not have as successful an outcome
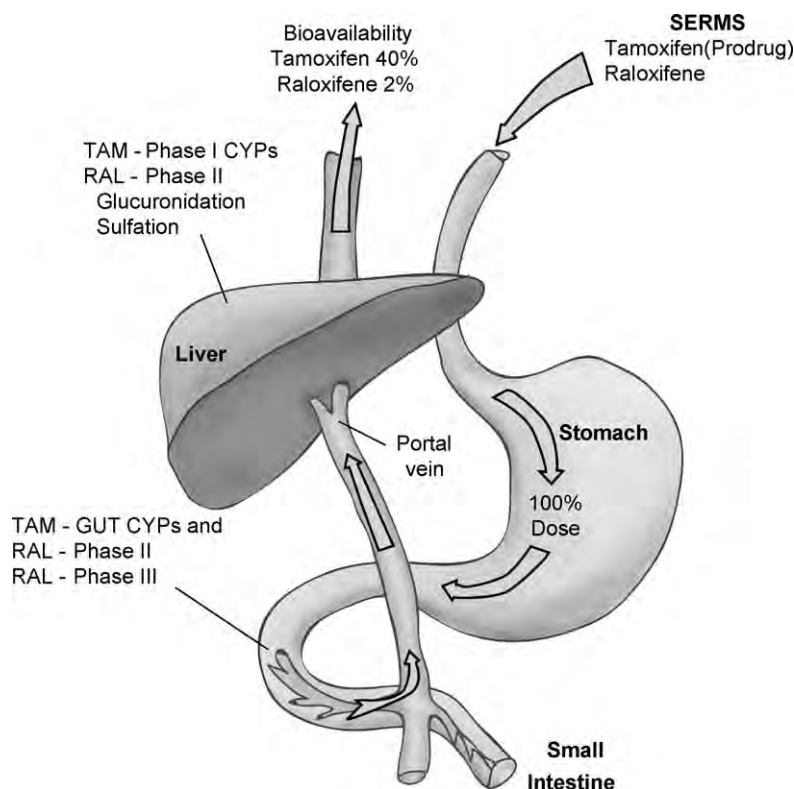
Fig. 1 – The stylized representation of the absorption of two selective estrogen receptor modulators (SERMS) tamoxifen (TAM) or raloxifene (RAL) into the circulation as bioactive molecules. The polyphenolic SERM raloxifene must transverse phase II and phase III obstacles in the gut and the liver to get into the general circulation. This results in very little of the ingested drug being bioavailable at target sites. In contrast, tamoxifen is extremely lipophilic and 98% protein bound to serum albumin. This extends the duration of action of tamoxifen because phase II metabolism to phenolic compounds is retarded.

with tamoxifen therapy. Alternatively, women who have a normal enzyme may make high levels of the potent antiestrogen endoxifen and experience hot flashes. As a result, these women may take selective serotonin reuptake inhibitors (SSRIs) to ameliorate hot flashes but there are potential pharmacological consequences to this strategy. Some of the SSRIs are metabolitically altered by the *CYP2D6* enzyme product [39]. It is therefore possible to envision a drug interaction whereby SSRIs block the metabolic activation of tamoxifen.

This article will describe the scientific twists and turns that tamoxifen and its metabolites have taken over the past 30 years. The story is naturally dependent on the fashions in therapeutic research at the time. What seems obvious to us as a successful research strategy today, with millions of women taking tamoxifen, was not so 30 years ago at the beginning when the clinical community and pharmaceutical industry did not see "antihormones" as a priority at all for drug development [25]. In 1972, tamoxifen was declared an orphan drug with no prospects [2].

## 3. Basic mechanisms of tamoxifen metabolism

The original survey of the putative metabolites of tamoxifen was conducted in the laboratories of ICI Pharmaceuticals Divi-

sion and published in 1973 [40]. A number of hydroxylated metabolites were noted (Fig. 3) following the administration of $^{14}$C labeled tamoxifen to various species (rat, mouse, monkey, and dog). The major route of excretion of radioactivity was in the feces. The rat and dog were used to show that up to 53% of the radioactivity derived from tamoxifen was excreted via the bile and up to 69% of this was reabsorbed via a enterohepatic recirculation until eventual elimination occurs [40]. The hydroxylated metabolites are excreted as glucuronides. However, no information about their biological activity was available until the finding that 4-hydroxytamoxifen had a binding affinity for the ER equivalent to 17β estradiol [32]. Similarly, 3,4-dihydroxytamoxifen (Fig. 3) bound to the human ER but interestingly enough, 3,4-dihydroxytamoxifen was not significantly estrogen-like in the rodent uterus despite being antiestrogenic [32].

Additional studies on the metabolism of tamoxifen in four women [41] identified 4-hydroxytamoxifen as the primary metabolite using a thin layer chromatographic technique to identify $^{14}$C labeled metabolites. This assumption, coupled with the potent antiestrogenic actions of 4-hydroxytamoxifen [32] and the conclusion that it was an advantage, but not a requirement for tamoxifen to be metabolically activated [33,42] seemed to confirm the idea that 4-hydroxytamoxifen was the active metabolite that bound in rat estrogen target tissues to block estrogen action [34]. However, the origi-
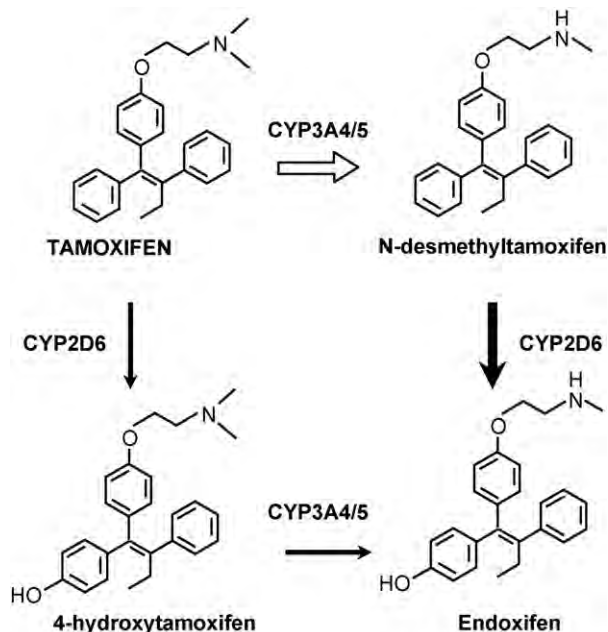
**Fig. 2 – The metabolic activation of tamoxifen to phenolic metabolites that have a high binding activity for the human estrogen receptor. Both 4-hydroxytamoxifen and endoxifen are potent antiestrogens _in vitro_.**

nal analytical methods used to identify 4-hydroxytamoxifen as the major metabolite in humans were flawed [43] and subsequent studies identified N-desmethyltamoxifen (Fig. 4) as the major metabolite circulating in human serum [44]. The metabolite was found to be further demethylated to N-desdimethyltamoxifen (metabolite Z) [45] and then deaminated to metabolite Y, a glycol derivative of tamoxifen [46,47].

The metabolites (Fig. 4) that are not hydroxylated at the 4 position of tamoxifen (equivalent to the three phenolic hydroxyl of estradiol) are all weak antiestrogens that would each contribute to the overall antitumor actions of tamoxifen at the ER based on their relative binding affinities for the ER and their actual concentrations locally.

At the end of the 1980s the identification of another metabolite tamoxifen 4-hydroxy N-desmethyltamoxifen in animals [48] and man [35,36] was anticipated but viewed as obvious and uninteresting. The one exception that was of interest was metabolite E (Fig. 3) identified in the dog [40]. This phenolic metabolite without the dimethylaminoethyl side chain is a full estrogen [47,49]. The dimethylaminoethoxy side chain of tamoxifen is necessary for antiestrogenic action [49].

It is not a simple task to study the actions of metabolites _in vivo_. Problems of pharmacokinetics, absorption and subsequent metabolism all conspire to confuse the interpretation of data. Studies _in vitro_ using cell systems of estrogen target tissues were defined and refined in the early 1980s to create an understanding of the actual structure–function relationships of tamoxifen metabolites. Systems were developed to study the regulation of the prolactin gene in primary cultures of immature rat pituitary gland cells [42,50] or cell replication in ER positive breast cancer cells [51–54]. Overall, these models were used to describe the importance of a phenolic hydroxyl to tether a triphenylethylenes appropriately in the ligand-binding domain of the ER and to establish the appropriate positioning of an "antiestrogenic" side chain in the "antiestrogen region" of the ER [50] to modulate gene activation and growth [42,50,55–58]. These structure–function studies, that created hypothetical models of the ligand-ER/complex, were rapidly advanced with the first reports of the X-ray crystallography of the estrogen, 4-hydroxytamoxifen [59] or raloxifene ER [60] complexes. The ligand–receptor protein interaction
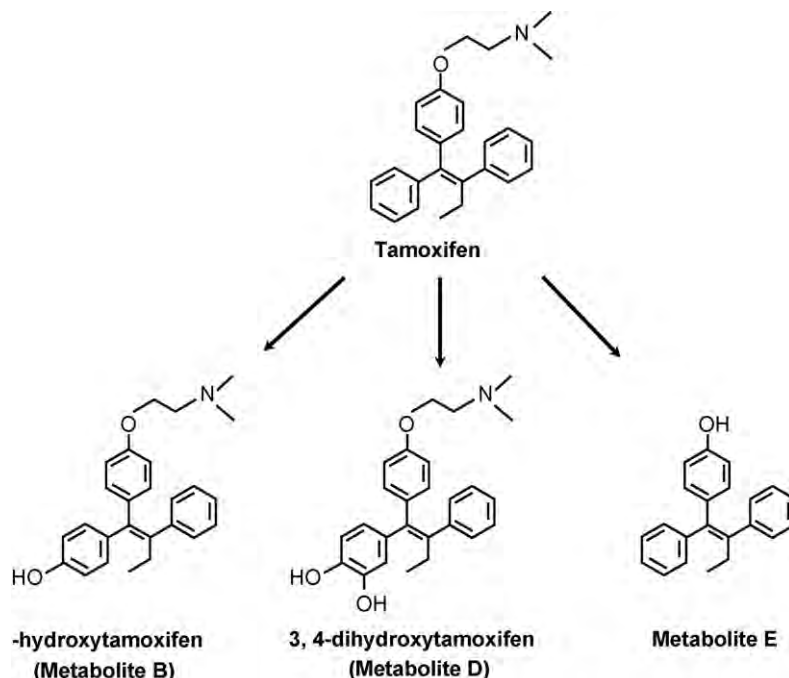


**Fig. 3 – The original hydroxylated metabolites of tamoxifen noted in animals by Fromson et al. [40].**
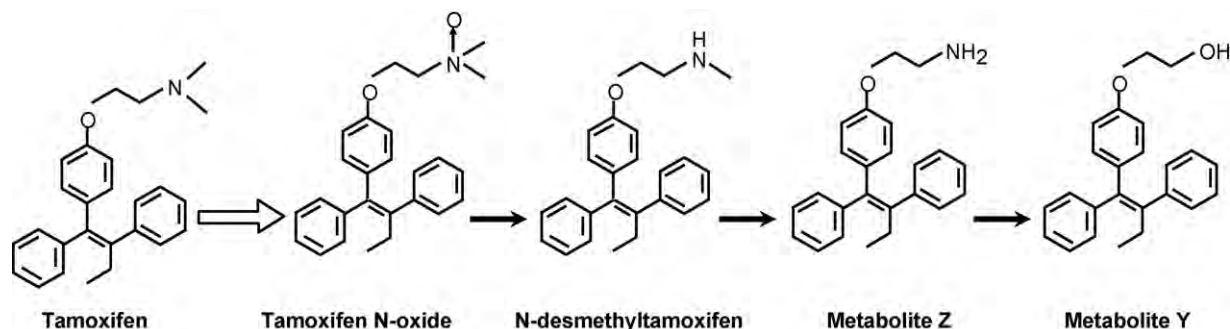
**Fig. 4 – The serial metabolic demethylation and deamination of the antiestrogenic side chain of tamoxifen. Each of the metabolites is a weak antiestrogen with poor binding affinity for the estrogen receptor.**

was subsequently interrogated by examining the interaction of the specific amino acid, asp 351 with the antiestrogenic side chain of the ligand [61]. A mutation was found as the dominant ER species in a tamoxifen-stimulated breast tumor grown in athymic mice [61,62]. The structure–function relationships studies, that modulated estrogen action at a transforming growth factor alpha gene target, demonstrated that the ligand shape would ultimately program the shape of the ER complex in a target tissue [30,63–65]. This concept is at the heart of metabolite pharmacology and is required to switch on and switch off target sites around the body. The other piece of the mechanism of SERMs puzzle that was eventually solved was the need for another player to partner with the ER complex. Coactivators [66] can enhance the estrogen-like effects of compounds at a target site [67]. However, in the early 1990s, the molecular and clinical use of this knowledge with the development and application of SERMs was in the future [68].

The urgent focus of translational research in the early 1990s was to discover why tamoxifen was a complete carcinogen in rat liver [69,70] and to determine whether there was a link between metabolism and the development of endometrial cancer noted in very small but significant numbers of postmenopausal women taking adjuvant tamoxifen [71,72].

All interest in the metabolism of tamoxifen focused on the production of DNA adducts [73] that were responsible for rat liver carcinogenesis and, at the time, believed to be poten-

tially responsible for carcinogenesis in humans [74]. Although many candidates were described [75–78], the metabolite found to be responsible for the initiation of rat liver carcinogenesis is α-hydroxytamoxifen [79–83] (Fig. 5). α-Hydroxytamoxifen has been resolved into R-(+) and S-(−) enantiomers. Metabolism by rat liver microsomes gave equal amounts of the two forms, but in hepatocytes the R form gave 8× the level of DNA adducts as the S form. As both had the same chemical reactivity towards DNA, Osborne et al. [84] suggested that the R form was a better sulfotransferase substrate. This enzyme is believed to catalyze DNA adduct formation. Subsequently, Osborne et al. [85] conducted studies with alpha-hydroxy-N-desmethyltamoxifen; the R-(+) gave 10× the level of adducts in rat hepatocytes as the S-(−).

There were reasonable concerns that the hepatocarcinogenicity of tamoxifen in rats would eventually translate to humans but fortunately this is now known to be untrue [86]. The demonstration of carcinogenesis in the rat liver appears to be related to poor DNA repair mechanisms in the inbred strains of rats. In contrast, it appears that the absence of liver carcinogenesis in women exposed to tamoxifen [87] is believed to result from the sophisticated mechanisms of DNA repair inherent in humans cells.

It is clear from this background about the early development of tamoxifen and the fact that tamoxifen was considered to be such a safe drug in comparison to other cytotoxic agents
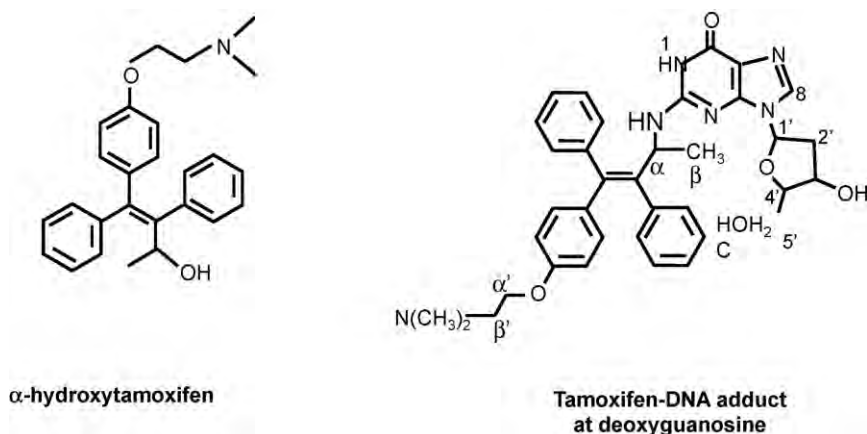


**Fig. 5 – The putative metabolite of tamoxifen, α-hydroxytamoxifen that produces DNA adducts through covalent binding to deoxyguanosine.**
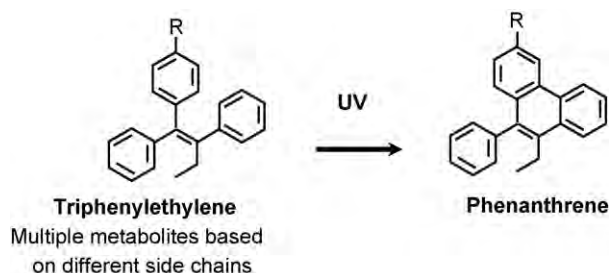
**Fig. 6 – The UV activation of a triphenylethylenes to a florescent phenanthrene. This basic reaction is exploited in the detection of serum tamoxifen levels.**

used in therapy during the 1970s and 1980s, that there was little enthusiasm for in-depth studies of tamoxifen metabolism. However, this perspective was to change in the 1990s with the widespread use of tamoxifen as the gold standard for the treatment and prevention of breast cancer. Questions needed to be addressed: (1) what happens to tamoxifen in patients? and (2) can improvements be made to the molecule?.

## 4.  Clinical pharmacology

A number of analytical techniques are available to evaluate blood levels of tamoxifen and its metabolites once the drug is absorbed. The early method of thin layer chromatography, and the current method of high performance liquid chromatography (HPLC) both depend on the conversion of the triphenylethylenes to fluorescent phenanthrenes for their detection (Fig. 6). The original description of the reaction [88] was successfully adapted [89] to identify tamoxifen, N-desmethyltamoxifen and 4-hydroxytamoxifen in plasma samples.

Subsequent improvements were made [90] but the method significantly underestimated phenolic metabolites (4-hydroxytamoxifen) and had no internal standardization. In contrast, a method of post-column fluorescence activation [91] or preliminary purification from interfering substance using a Sep-Pack C18 cartridge (Waters Association, Milford, MA) [92] with internal standardization considerably improved accuracy. The detection of tamoxifen metabolites in serum was further improved by Lien et al. [93] and recently by Lee et al. [94] who adapted the methods [95,96] developed to perform "on line" extraction and post-column cyclization. Using this methodology the limits of detection for 4-hydroxy tamoxifen and endoxifen are 0.5 and 0.25 ng/ml, respectively [97]. Since there was such initial controversy about the identification of metabolites in patient serum, it is perhaps important to describe the validation of 4-hydroxy-desmethyltamoxifen as a metabolite of tamoxifen in patients. Tamoxifen metabolites were investigated in a 57-year-old female patient receiving tamoxifen treatment [35]. Two major chromatographic peaks were identified in bile following treatment with β-glucuronidase. On major peak co-eluted with 4-hydroxytamoxifen but the second peak was proven to be 4-hydroxy-N-desmethyltamoxifen using (a) co-elution with an authentic standard on reversed-phase chromatography

and formation of fluorescent derivative by cyclization; (b) the detection of a molecular ion (M + l)+ of 374 m/2 as determined by liquid chromatography–mass spectrometry; and (c) a fragmatogram identical to that of the authentic standard, obtained by mass spectrometry. Subsequent refinement of the technology improved detection for identification of 4-hydroxy-N-desmethyltamoxifen in human serum, tissues [36] and rat tissues [93].

Studies confirm that tamoxifen is 98% bound to serum albumin which ultimately creates a long biological half-life (plasma half-life 7 days) [93]. A single oral dose of 10 mg tamoxifen (half the daily dose) produces peak serum levels of 20–30 ng of tamoxifen/ml within 3–6 h but it must be stressed that patient variation is very large [98]. Nevertheless, continuous therapy with either 10 mg bid [98] or 20 mg bid [99] produces steady state levels within 4 weeks. Blood levels of tamoxifen can average around 150 ng/ml for 10 mg tamoxifen bid and 300 ng/ml for 20 mg tamoxifen bid. A strategy of using loading doses [98,100] to elevate blood levels rapidly has not produced any therapeutic benefit.

Overall, the results from the metabolic studies with tamoxifen during the 1970s and 1980s did not help clinicians to use tamoxifen more effectively. The structures of metabolites were in fact used as leads to create new molecules for clinical development.

## 5.  Metabolic mimicry

The demonstration [32] that the class of compounds referred to as nonsteroidal antiestrogens were metabolically activated to compounds with high binding affinity for the ER created additional opportunities for the medicinal chemists within the pharmaceutical industry to develop new agents. This was particularly true once the nonsteroidal antiestrogens were recognized to be SERMs [101–103] and had applications not only for the treatment and prevention of breast cancer but also as potential agents to treat osteoporosis and coronary heart disease [104,105]. The reader is referred to other recent review articles to obtain further details of new medicines under investigation [104,105] but some current examples are worthy of note and will be mentioned briefly. Compounds of interest that have their structural origins as metabolites from nonsteroidal antiestrogens are summarized in Fig. 7. Raloxifene is an agent that originally was destined to be a drug to treat breast cancer but it failed in that application [106]. It appears that the pharmacokinetics and bioavailability of raloxifene are a challenge. Only about 2% of administered raloxifene is bioavailable [1] but despite this, the drug is known to have a long biological half-life of 27 h. The reason for this disparity is that raloxifene is a polyphenolic drug that can be glucuronidated and sulfated by bacteria in the gut so the drug cannot be absorbed [107,108]. This phase II metabolism in turn controls enterohepatic recirculation and ultimately impairs the drug from reaching and interacting with receptors in the target. This concern has been addressed with the development of the long-acting raloxifene derivative arzoxifene that is known to be superior to raloxifene as a chemopreventive in rat mammary carcinogenesis [109]. One of the phenolic groups (Fig. 7) is methylated to provide protection from phase II metabolism.
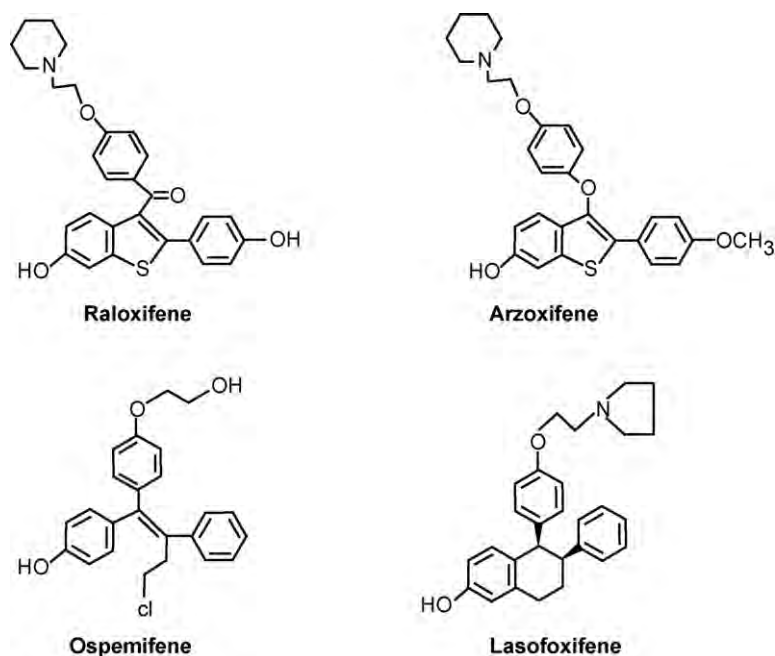
**Fig. 7 – The formulae of SERMs that have been developed based on the knowledge of the metabolic activation of tamoxifen (and nafoxidine, see text) as well as the metabolism of the antiestrogen side chain of tamoxifen to a glycol.**

Nevertheless, arzoxifene has not performed well as a treatment for breast cancer [110,111]; higher doses are less effective than lower doses. These data imply that effective absorption is impaired by phase III metabolism. That being said, the results of trials evaluating the effects of arzoxifene as a drug to treat osteoporosis, using lower doses, are eagerly awaited. Perhaps arzoxifene will be a better breast cancer preventive than a treatment.

Unfortunately, the bioavailability of phenolic drugs is also dependent on phase II metabolism to inactive conjugates in the target tissue. 4-Hydroxytamoxifen [32] is only sulfated by three of seven sulfotransferase isoforms whereas raloxifene is sulfated by all seven [112]. Maybe local phase II metabolism plays a role in neutralizing the antiestrogen action of raloxifene in the breast. Falany et al. [112] further report that SULT1E1, that sulfates raloxifene in the endometrium, is only expressed in the secretory phase. In contrast, 4-hydroxytamoxifen is sulfated at all stages of the uterine cycle.

Lasofoxifene is a diaryltetrahydronaphthalene derivative referred to as CP336156 [113] that has been reported to have high binding affinity for ER and have potent activity in preserving bone density in the rat [114,115]. The structure of CP336156 is reminiscent of the putative antiestrogenic metabolite of nafoxidine [116] that failed to become a breast cancer drug because of unacceptable side effects [117]. There are two disasterometiric salts of the chemical shown in Fig. 7. CP336156 is the l enantiomer that has 20 times the binding affinity for the ER as the d enantiomer. Studies demonstrate that the l enantiomer had twice the bioavailability of the d enantiomer. The authors [113] ascribed the difference to enantioselective glucuronidation of the d isomer. An evaluation of CP336156 in the prevention and treatment of rat mammary tumors induced by N-nitroso-N-methylurea shows activity similar to that of tamoxifen [118].

Ospemifene or deaminohydroxytoremifene is related to metabolite Y formed by the deamination of tamoxifen [47]. Metabolite Y has a very low binding affinity for the ER [47,119] and has weak antiestrogenic properties compared with tamoxifen. Ospermifene is a known metabolite of toremifene (4 chlorotoremifene) but unlike tamoxifen, there is little carcinogenic potential in animals [120]. It is possible that the large chlorine atom on the 4 position of toremifene and ospermifene reduces α hydroxylation to the ultimate carcinogen related to α hydroxy tamoxifen (Fig. 6). Deaminohydroxytoremifene has very weak estrogenic and antiestrogenic properties in vivo [121] but demonstrates SERM activity in bone and lowers cholesterol. The compound is proposed to be used as a preventative for osteoporosis. Preliminary clinical data in healthy men and postmenopausal women demonstrate pharmacokinetics suitable for daily dosing between 25 and 200 mg [122]. Interestingly enough, unlike raloxifene, ospermifene has a strong estrogen-like action in the vagina but neither ospermifene nor raloxifene affect endometrial histology [123,124]. Overall, the goal of developing a bone specific agent is reasonable, but the key to commercial success will be the prospective demonstration of the prevention of breast and endometrial cancer as beneficial side effects. This remains a possibility based on prevention studies completed in the laboratory [125,126].

## 6. Tamoxifen metabolism today

A comprehensive evaluation of the sequential biotransformation of tamoxifen has been completed by Desta et al. [38]. They used human liver microsomes and experiments with specifically expressed human cytochrome P450's to identify the prominent enzymes involved in phase I metabolism. Their

results are summarized in Fig. 2 with the relevant CYP genes indicated for the metabolic transformations. The authors make a strong case that N-desmethyltamoxifen, the principal metabolite of tamoxifen that accumulates in the body, is converted to endoxifen by the enzymatic product of CYP2D6. The CYP2D6 product is also important to produce the potent primary metabolite 4-hydroxytamoxifen but the metabolite can also be formed by the enzymatic products: CYP2B6, CYP2C9, CY2C19 and CYP3A4.

The CYP2D6 phenotype is defined as the metabolic ratio (MR) by dividing the concentration of an unchanged probe drug, known to be metabolized by the CYP2D6 gene product, by the concentration of the relevant metabolite at a specific time. These measurements have resulted in the division of the CYP2D6 phenotype in four metabolic classes: poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM) and ultrarapid metabolizes (UM). Over 80 different single nucleotide polymorphisms have been identified but there are inconsistencies in the precise definitions of the ascribing a genotype to a phenotype [127,128]. Bradford [128] and Raimundo et al. [129] have described the frequency of common alleles for CYP2D6. Pertinent to the current discussion of tamoxifen metabolism, the CYP2D6*4 allele [130] is estimated to have a frequency of 12–23% in Caucasians, 1.2–7% in black Africans and 0–2.8% in Asians [127,128]. A lower estimate of (<10%) of the PM phenotype is presented by Bernard et al. [131].

The molecular pharmacology of endoxifen has recently been reported [37,132,133]. Endoxifen and 4-hydroxytamoxifen were equally potent at inhibiting estrogen-stimulated growth of ER positive breast cancer cells MCF-7, T47D and BT474. Both metabolites are significantly superior in vitro to tamoxifen the parent drug. Additionally, the estrogen-responsive genes $pS_2$ and progesterone receptor were both blocked to an equivalent degree by endoxifen and 4-hydroxytamoxifen [132,133]. Lim et al. [133] have extended the comparison of endoxifen and 4-hydroxytamoxifen in MCF-

7 cells by comparing and contrasting global gene regulation using the Affymetrix U133A Gene Chip Array. There were 4062 total genes that were either up or down regulated by estradiol whereas, in the presence of estradiol, 4-hydroxytamoxifen or endoxifen affected 2444 and 2390 genes, respectively. Overall, the authors [133] demonstrated good correlation between RTPCR and select genes from the microarray and concluded that the global effects of endoxifen and 4-hydroxytamoxifen were similar.

Stearns et al. [97] and Jin et al. [134] have confirmed and significantly extended Lien's original identification of endoxifen and observation [35,36] that there are usually higher circulating levels of endoxifen than 4-hydroxytamoxifen in patients receiving adjuvant tamoxifen therapy. However, Flockhart's group [97] have advanced the pharmacogenomics and drug interactins surrounding tamoxifen therapy that should be a consideration in the antihormonal treatment of breast cancer.

The ubiquitous use of tamoxifen for the treatment of node negative women [135] during the 1990s, the use of tamoxifen plus radiotherapy following lumpectomy for the treatment of ductal carcinoma in situ (DCIS) [136] as well as the option to use tamoxifen for chemoprevention in high risk pre- and postmenopausal women [137] enhanced awareness of the menopausal side effects experienced by women when taking tamoxifen. Up to 45% of women with hot flashes grade them as severe [137] therefore there have been efforts to improve quality of life. Treatments with the SSRIs are popular [97,138,139] (Fig. 8). The SSRIs are twice as effective as the "placebo" effect at reducing menopausal symptoms in randomized clinical trials [138–140], so there is naturally an increased usage of SSRIs with long-term tamoxifen treatment to maintain compliance. Unfortunately, the metabolism of tamoxifen to hydroxylated metabolites [141–143] and the metabolism of SSRIs [39,144–147] both occur via the CYP2D6 gene product. Indeed Stearns et al. [97] showed that the SSRI inhibitor paroxetine reduced the levels of endoxifen during adjuvant tamoxifen
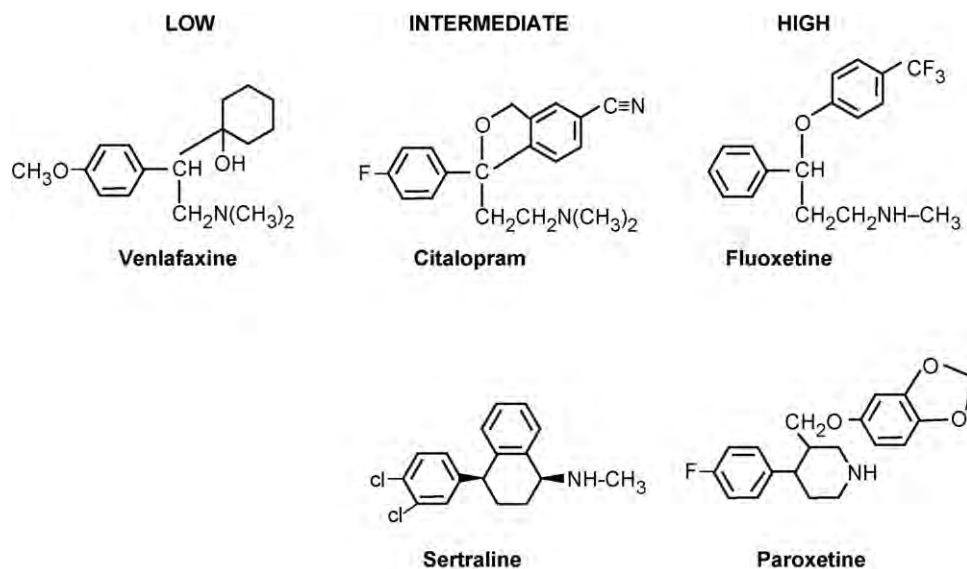


Fig. 8 – The structures of selective serotonin reuptake inhibitors (SSRIs) that have low intermediate or high affinity for the CYP2D6 enzyme system. High affinity binders for CYP2D6 block the metabolic activation of tamoxifen to endoxifen (Fig. 2).

therapy and endoxifen levels decrease by 64% in women with wild type *CYP2D6* enzyme. Patients were examined who were taking venlafaxine, sertraline, and paroxetine and compared with those women who were homozygotes for the *CYP2D6\*4/\*4* inactive genotype. Patients with the wild type gene who took the most potent inhibitor paroxetine had serum levels of endoxifen equivalent to the patients with the aberrant *CYP2D6* gene. In fact, the clinical data were consistent with the inhibition constants for the inhibition of *CYP2D6* by paroxetine (potent), fluoxetine, sertraline, citalopram (intermediate) and venlafaxine (weak) which are 0.05, 0.17, 1.5, 7 and 33 µmol/l, respectively.

The *CYP2D6* gene product that is fully functional (wild type) is classified as the *CYP2D6\*1*. A large number of alleles are associated with no enzyme activity or reduced activity. Conversely, high metabolizers can have multiple copies of the *CYP2D6* allele [31]. A recent study by Borges et al. [148] continues to expand our understanding of the detrimental effect of *CYP2D6* variants plus concomitant administration of SSRIs on endoxifen levels. But, it is the clinical correlations with tumor responses and side effects that are starting to provide clues about the importance of pharmacogenomics for tamoxifen to be optimally effective as a breast cancer drug.

## 7. Clinical correlations

The significance of genotyping on clinical outcomes of a tamoxifen trial have been addressed using paraffin-embedded tumor blocks from a North Central Center Treatment Group (NCCTG) trial NCCTG 89-30-52 [149]. The postmenopausal women with ER positive tumors received 5 years of adjuvant tamoxifen therapy. The tumor blocks were used to determine CY2D6 (*4 and *6) and *CYP3A5* (*3) and 17 buccal swabs were used to test the veracity of the tumor genotyping. The concordance rate for the buccal swabs was 100%. Overall, the *CYP3A5\*3* variant was not associated with any adverse clinical outcomes but the women with the *CYP2D6\*4/\*4* genotype had a higher risk of disease relapse but a lower incidence of side effects such as hot flashes [149]. The implication is that tamoxifen must be converted to endoxifen, a more potent antiestrogen.

In a follow up study [150] using the same database established for trial NCCTG 89-30-52, patient records were screened to determine the extent of SSRI prescribing. The goal was to establish the combined effect of genotyping and SSRI inhibition of the *CYP2D6* enzyme. Overall, the authors [150] concluded that a mutated *CYP2D6* gene or the inadvertent use of SSRIs that inhibit the *CYP2D6* enzyme product are independent predictors of breast cancer outcomes for postmenopausal women with breast cancer taking tamoxifen. In a recent complimentary study, Mortimer et al. [151] demonstrated that hot flashes were a strong predictor of positive outcomes for adjuvant tamoxifen treatment.

Although all of the current emphasis has been on the biological effects of tamoxifen in patients with the *CYPD6\*4* variant, studies of *CYP3A5\* 1* and *\*3 1A1 \*1* and 2 and *UGT2B15 \** and *\*2* have been undertaken and compared with car-

riers of *CYP2D6\*4*. In contrast to the studies of Goetz et al. [149], patients who carry the *SULT1A1\*1*, *CYP2D7\*4* and *CYP3A5\*3* alleles, and would be predicted to give rise to lower concentrations of metabolites with high affinity for the ER, might actually benefit from tamoxifen [152–155]. No differences were noted between genotypes *CYP2D6*, *SULT1A1* or *UGT 2B15* and tamoxifen treatment but Wegman et al. [155] claim that genetic variants of CYP3A5 may predict response to tamoxifen. Clearly, reasons for the different conclusions need to be advanced. The hypothesis that variants of metabolizing enzymes can affect patient outcomes for the treatment of breast cancer must now be addressed in large populations and with prospective studies.

## 8. Conclusions

Overall, the study of tamoxifen metabolism has provided important clues which guided medicinal chemists to synthesize and develop new medicines. The study of metabolites has also provided valuable insight into the mechanism of action of SERMs at their target the ER. However, it is the recent research on the value of genotyping *CYPs* in breast cancer patients to improve response rates to tamoxifen therapy that is showing important promise. Genotyping patients for *CYP2D6* appears to be valuable to exclude the suboptimal use of tamoxifen in select individuals. Additionally, and perhaps more importantly, an effect of SSRIs on the blood levels of endoxifen has raised the possibility that the cheap and effective veteran tamoxifen could be targeted further to select populations of women to improve response rates. Avoiding SSRIs with a high affinity for *CYP2D6* gene product could improve tamoxifen's efficacy. Since tamoxifen is still the antihormonal treatment of choice for premenopausal patients and the only choice for breast cancer risk reduction in premenopausal women, then genotyping from buccal swabs appears to be a cheap and effective way of ensuring that tamoxifen is used to treat the appropriate woman.

It is necessary, however, to close on a note of caution. Very few patients have been studied to create definitive guidelines. That being said, the task of proving the value of these tantalizing clues and hypotheses is the responsibility of clinicians to organize prospective clinical trials or at least there must be investment in the further analysis of archival material from randomized trials. The value of committing resources to establish hypothesis as fact is clear. An important cheap medicine should potentially be given only to women who will benefit from it. Indeed, it may be the role of *CYP2D6* in tamoxifen metabolism that is creating the small but significant advantage of aromatase inhibitors versus tamoxifen in postmenopausal women [26,27]. Again, this can be tested as the tumor blocks and patient records could be reviewed to determine genotyping and whether SSRIs were used. It would be remarkable to discover that the pharmacology of tamoxifen is undermining activity rather than the current view that aromatase inhibitors were better medicines because they have, unlike the SERMs, no estrogen-like actions at the level of the tumor.

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Glutathione depletion sensitizes hormone-independent human breast cancer cells to estrogen-induced apoptosis

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**Abstract**: Glutathione (GSH) is a naturally occurring tripeptide whose nucleophilic and reducing properties play a central role in metabolic pathways, as well as in the antioxidant system of aerobic cells. In many cell systems, pharmacological GSH depletion with the GSH biosynthesis inhibitor L-buthionine-S,R-sulfoximine (BSO) leads to cell death and highly sensitizes tumor cells to apoptosis induced by standard chemotherapeutic agents. Our laboratory has previously reported the development of a unique subclone of the MCF-7 human breast cancer cell line, named MCF-7:5C, which grows maximally in the absence of endogenous estrogen but undergoes apoptosis in the presence of physiologic concentrations of 17β-estradiol ($E_2$) via activation of the mitochondrial cell death pathway (Lewis et al., JNCI 2005). In the present study, we have identified and characterized another subclone of the MCF-7 cell line, named MCF-7:2A, which undergoes apoptosis in the presence of BSO plus $E_2$ but not $E_2$ alone. Exposure of MCF-7:2A cells to 1 n*M* $E_2$ or 100 μ*M* BSO for 48-96 hours did not produce cell death, however, the combination treatment produced a dramatic increase (7-fold) in apoptosis which was evidenced by Annexin V-PI and TUNEL staining. Microarray studies revealed that glutathione synthetase (GSS) and glutathione peroxidase 2 (GPX2) genes were overexpressed by 6-fold and 40-fold, respectively, in MCF-7:2A cells compared to hormone-responsive MCF-7 cells. Cellular GSH levels were also significantly ($p<.0001$) elevated in MCF-7:2A cells compared to MCF-7 cells and BSO almost completely depleted GSH. Induction of apoptosis by the combination treatment of $E_2$ plus BSO was also evidenced by changes in Bcl-2, Bcl-xl, and Bax expression, mitochondrial membrane potential and cytochrome c release, poly(ADP-ribose)polymerase (PARP) cleavage and caspase 9 and caspase-7 activation. The combination treatment also markedly reduced phosphorylated p38 MAPK and phosphorylated JNK levels in MCF-7:2A cells. In addition, blockade of the JNK pathway using the inhibitor SP600125 almost completely attenuated the apoptotic effect of $E_2$ plus BSO thus suggesting an important role for JNK in mediating the apoptotic effects of $E_2$ and BSO in MCF-7:2A cells. Our data indicates that GSH participates in apoptosis in hormone-resistant breast cancer cells and that depletion of this molecule may be critical in predisposing these cells to apoptotic cell death.

**GPR30 modulates estrogen-stimulated proliferation of breast and endometrial cancer cells by regulating estrogen receptor alpha homeostasis**

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GPR30 is a 7-transmembrane spanning G protein-coupled receptor that has been identified as a novel $17\beta$-estradiol ($E_2$) -binding protein structurally distinct from the classical estrogen receptors $\alpha$ and $\beta$ (ER$\alpha$ and ER$\beta$). Both classical ERs and GPR30 can mediate rapid $E_2$-induced non-genomic signaling which activates many of the same cellular effectors such as PI3K and MAPK, however the pathways differ. Additionally, ER$\alpha$ and GPR30 expression by immunohistochemistry have been shown to correlate in clinical breast cancer. We therefore investigated whether GPR30 and ER$\alpha$ share a functional relationship in estrogen-responsive human MCF-7 breast and ECC-1 endometrial cancer cells. RNAi-mediated depletion of GPR30 significantly inhibited proliferation of both MCF-7 and ECC-1 cells, as did ER$\alpha$ knockdown. RNAi-mediated knockdown of GPR30 led to a concomitant decrease in ER$\alpha$ expression, and conversely, depletion of ER$\alpha$ led to similarly decreased GPR30 expression. $E_2$ differentially regulated GPR30 expression in a cell type-dependent manner; E2 down-regulated GPR30 expression in MCF-7 cells while $E_2$ up-regulated GPR30 expression in ECC-1 cells. However, E2 down-regulated ER$\alpha$ expression in both cell types. Thus, homeostasis of GPR30 and ER$\alpha$ expression were coordinated, but $E_2$-mediated regulation of GPR30 and ER$\alpha$ expression were not coordinated. GPR30 depletion also led to inhibition of $E_2$-stimulated estrogen response element-regulated transcriptional activity using luciferase reporter genes, and blocked $E_2$-induction of progesterone receptor mRNA levels by real-time PCR. This decreased transcriptional activity was likely the result of GPR30 depletion causing a parallel decrease in ER$\alpha$ expression. However, GPR30 depletion altered expression of some $E_2$-regulated genes differently than had ER$\alpha$ depletion, such as with EGFR. Therefore, GPR30 can affect $E_2$-stimulated growth by regulating ER$\alpha$ expression and hence, modulating expression of at least some ER$\alpha$-target genes. Mining of the Netherlands Cancer Institute microarray gene expression data set of 295 breast cancers validated that GPR30 and ER$\alpha$ RNA expression correlate, and further indicated that GPR30 expression was highest in the Luminal B subtype, which is associated with poor prognosis. Therefore, GPR30 regulates ER$\alpha$ homeostasis, likely plays an important role in cancer, and represents a promising target for therapeutic intervention.

Glutathione depletion sensitizes hormone-independent human breast cancer cells to estrogen-induced apoptosis

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**Abstract**: Glutathione (GSH) is a naturally occurring tripeptide whose nucleophilic and reducing properties play a central role in metabolic pathways, as well as in the antioxidant system of aerobic cells. In many cell systems, pharmacological GSH depletion with the GSH biosynthesis inhibitor L-buthionine-S,R-sulfoximine (BSO) leads to cell death and highly sensitizes tumor cells to apoptosis induced by standard chemotherapeutic agents. Our laboratory has previously reported the development of a unique subclone of the MCF-7 human breast cancer cell line, named MCF-7:5C, which grows maximally in the absence of endogenous estrogen but undergoes apoptosis within 24-48 hours of 17$\beta$-estradiol (E$_2$) treatment via activation of the mitochondrial cell death pathway (Lewis et al, *J Natl Cancer Inst*. 2005; 97:1746-59). In the present study, we have identified and characterized another subclone of the MCF-7 cell line, named MCF-7:2A, which unlike the MCF-7:5C cells, undergoes apoptosis only in the presence of E$_2$ plus BSO but not E$_2$ alone. Exposure of MCF-7:2A cells to 1 n$M$ E$_2$ or 100 $\mu M$ BSO for 48-96 hours did not produce cell death, however, the combination treatment produced a dramatic increase (7-fold) in apoptosis which was evidenced by Annexin V-PI and TUNEL staining. Microarray studies revealed that glutathione synthetase (GSS) and glutathione peroxidase 2 (GPX2) genes were overexpressed by 6-fold and 40-fold, respectively, in MCF-7:2A cells compared to hormone-responsive MCF-7 cells. Cellular GSH levels were also significantly ($p<.0001$) elevated in MCF-7:2A cells compared to MCF-7 cells and BSO almost completely depleted GSH. Induction of apoptosis by the combination treatment of E$_2$ plus BSO was also evidenced by changes in Bcl-2, Bcl-xl, and Bax expression, mitochondrial membrane potential and cytochrome c release, poly(ADP-ribose)polymerase (PARP) cleavage and caspase 9 and caspase-7 activation. The combination treatment also markedly reduced phosphorylated p38 MAPK and upregulated phosphorylated JNK levels in MCF-7:2A cells. In addition, blockade of the JNK pathway using the inhibitor SP600125 almost completely attenuated the apoptotic effect of E$_2$ plus BSO thus suggesting an important role for JNK in mediating the apoptotic effects of E$_2$ and BSO in MCF-7:2A cells. In conclusion, our data indicates that GSH participates in apoptosis in hormone-resistant breast cancer cells and that depletion of this molecule may be critical in predisposing these cells to apoptotic cell death.

# Genomic evolution of endocrine-resistant breast cancer cell lines reveals molecular aberrations consistent with biological phenotype.

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Suppression of estrogen synthesis using aromatase inhibitors is highly effective in the treatment of postmenopausal women with estrogen-receptor alpha (ERalpha)-positive breast cancer. Third generation aromatase inhibitors are superior to adjuvant tamoxifen resulting in improved disease-free survival and a lower incidence of side effects. Unfortunately, one of the consequences of long-term estrogen deprivation or exhaustive endocrine therapy is the development of drug resistance. The Jordan laboratory and others have shown that the acquisition of resistance to long term estrogen deprivation or to selective estrogen receptor modulators (SERMs) in breast cancer cells is accompanied by an increase in malignant cell behavior. We have conducted an array-based genomic study to elucidate molecular mechanisms associated with development of endocrine resistance.

MCF-7:5C and MCF-7:2A are two ERalpha-positive human breast cancer cell lines derived from long-term estrogen deprivation of wild type hormone-dependent MCF-7 cells. MCF-7:TAM2 and MCF-7:RAL2 are also ERalpha-positive derivatives of MCF-7 that are resistant to the SERMs Tamoxifen and Raloxifene respectively.

Whole genome expression and array-based comparative genomic hybridization (aCGH) analysis were performed on each endocrine resistant cell line, compared to the parental MCF-7 cells. Unsupervised hierarchical clustering of global gene expression changes revealed a complex pattern of overlapping and distinct transcriptional changes. Analysis of aCGH profiles indicated both common and unique chromosomal breaks, as well as shared and unique regions of DNA gain and loss. Thus, the biological divergence of each cell line was apparent by aCGH and gene expression profiling. This suggests that long-term selective pressure exerted on MCF-7 cells results in a significant degree of genomic evolution, which contributes to observed patterns of gene expression, and presumably, biological behavior. Interestingly, amplification of the ERalpha gene (ESR1) was associated with resistance to long-term estrogen deprivation but not resistance to SERMs. Moreover, we observed several repeated genomic and transcriptional aberrations associated with long-term estrogen deprivation. For example, chromosomal regions harboring ESR1, BRCA1 and CDK4 genes are all amplified and overexpressed in MCF-7:5C and MCF-7:2A cells. Preliminary gene ontology analysis of genes differentially expressed by both MCF-7:5C and MCF-7:2A highlighted deregulated AKT signaling and cell cycle control. Elevated phospho-AKT was subsequently validated by western blot analysis. Our preliminary analysis suggests that biological drivers of endocrine resistance in each cell line model can be identified using bioinformatic approaches. We are currently prioritizing the likely molecular drivers of endocrine resistant cell line by comparison and extraction from both genomic and gene expression data.

**Comparative Gene Expression Profiling to Identify Unifying and Selective Pathways Involved in Tamoxifen, Raloxifene, and Aromatase Inhibitor-resistant Breast Cancer Xenograft Tumors**

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We have developed multiple xenograft breast cancer models of antihormone resistance to the selective estrogen receptor modulators (SERMs) tamoxifen (TAM) and raloxifene (RAL), and to estrogen deprivation as a surrogate for aromatase inhibitors (AIs). Using these models, we have defined Phase I and Phase II antihormonal resistance based on their growth responsiveness to $17\beta$-estradiol ($E_2$). Phase I SERM-resistant (*i.e.* MCF-7/RAL1) tumors are growth stimulated in response to either SERMs or $E_2$, whereas Phase II SERM (MCF-7/RAL2 and MCF-7/TAM2) and AI-resistant (MCF-7/5C) tumors paradoxically undergo $E_2$-induced regression due to apoptosis. In the current study, we compared gene expression profiles across these antihormone-resistant breast cancer models to identify unifying and selective pathways involved in their etiology, and to identify genes involved in this newly discovered mode of apoptotic action of $E_2$. Gene expression profiling was conducted using both Agilent 22k Human 1A (V2) Oligo Microarrays and Affymetrics Human U133 Plus 2.0 Arrays. Each tumor model showed distinct patterns of gene expression, however, hierarchical clustering showed that the Phase 2 tumors grouped together, validating our phenotypic classification. Differentially expressed genes were filtered for those genes that were commonly deregulated in both Phase I and II resistant tumors compared to wild-type MCF-7/E2 tumors, for those genes selectively associated with Phase II resistance, and for those genes differentially regulated by $E_2$ in the Phase II MCF-7/5C tumors versus wild-type tumors. In both Phase I and II resistant tumor types, nuclear receptor interacting protein 1 (NRIP1, RIP140), a corepressor of estrogen receptor $\alpha$ (ER$\alpha$) was consistently down-regulated, while mucin 1 (MUC1), which stabilizes and activates ER$\alpha$, was consistently up-regulated. Together, these changes in expression could enhance ER$\alpha$ activities in antihormone-resistant tumors. Other examples of genes coordinately deregulated in both Phase I and II resistant tumor types include chemokine receptor 4 (CXCR4), BCL2-associated anthanogene 1 (BAG1), immediate early response 3 (IER3), and WW domain containing oxidoreductase (WWOX). Examples of genes which were differentially regulated by $E_2$ in the Phase II MCF-7/5C tumors versus wild-type tumors include CCAAT/enhancer binding protein delta (CEBPD), SIN3 homolog B (SIN3B), and G protein-coupled receptor 30 (GPR30). GPR30 is a 7-transmembrane spanning protein that binds $E_2$ and can mediate rapid $E_2$-induced non-genomic signaling events.

We are examining molecular pathways indicated by the gene expression changes to understand mechanisms associated with Phase 1 and Phase 2 antihormone resistance. Currently, we are investigating a potential functional relationship between GPR30 and ER$\alpha$.

$E_2$-induced apoptosis in antihormone resistant breast cancer has not yet been widely recognized, but could be exploited by developing a novel treatment based on short-term, low-dose estrogen for patients who fail exhaustive endocrine therapy.

**The evolution of drug resistance to antihormonal therapy exposes a vulnerability in breast cancer**

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The ubiquitous application of selective estrogen receptor modulators (SERMs) and aromatase inhibitors for the treatment and prevention of breast cancer has created a significant advance in patient care. However, the consequences of prolonged treatment with antihormonal therapy is the development of drug resistance. Nevertheless, the systematic description model of drug resistance to SERMs and aromatase inhibitors has resulted in the discovery of a vulnerability in tumor homeostasis that can be exploited to improve patient care. Laboratory studies of exhaustive antihormonal therapy demonstrate that there are at least two phases of resistance to SERMs (tamoxifen and raloxifene) and to estrogen withdrawal (aromatase inhibitors). In Phase I drug resistance, estrogen or a SERM promote tumor growth, but in Phase II drug resistance, estrogen induces apoptosis. Understanding of the new biology of estrogen action has clinical relevance. It is clear that drug resistance to antihormones evolves so that eventually the cells change to create novel signal transduction pathways for enhanced estrogen (GPR30 plus ER) sensitivity, a reduction in progesterone receptor production, and an increased metastatic potential. We have initiated a major collaborative program of genomics and proteomics to use our laboratory models to map the mechanisms of subcellular survival and apoptosis in breast cancer. The laboratory program is integrated with a clinical program that seeks to determine a minimum dose of estrogen necessary to create objective responses in patients who have succeeded and failed two consecutive antihormonal therapies. Once our program is complete, a new knowledge will be available to translate the clinical care for the long term maintenance for patients on antihormonal therapy.

**Single Arm Phase II Study of Pharmacologic Dose Estrogen in Postmenopausal Women with Hormone Receptor-Positive Metastatic Breast Cancer After Failure of Sequential Endocrine Therapies**

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Breast cancer continues to be the most common malignancy affecting women. Although great strides have been made in the treatment and cure of early stage breast cancer, metastatic breast cancer remains incurable resulting in 40,000 deaths per year in the United States. Approximately two-thirds of all breast cancers contain the estrogen receptor (ER) and/or progesterone receptor (PgR) and are termed hormonally sensitive disease. A significant proportion of these hormonally sensitive breast cancers are dependent upon estrogenic stimulation for survival and growth. Historically, various techniques employing estrogen deprivation have been utilized to treat hormonally sensitive breast cancer; however, some of these tumors will ultimately become resistant to anti-estrogen treatment. The mechanism(s) of anti-estrogen resistance in initially estrogen responsive tumors has not been elucidated. Pre-clinical data suggest that estrogen sensitive breast cancers exposed to long-term estrogen deprivation as a result of antiestrogen treatment such as tamoxifen will evolve to no longer be responsive to such treatment, and then paradoxically become stimulated to regrow during treatment. These long-term estrogen deprived tumors exhibit increased levels of apoptosis, and may be hypersensitive to the effects of estrogen in this particular setting.

To further explore the mechanisms of *in vivo* anti-estrogen resistance, a single arm phase 2 clinical trial will be performed to evaluate the clinical response rate to pharmacologic dose estrogen (Estrace) treatment in post-menopausal estrogen receptor positive patients with metastatic breast cancer. Eligible patients will have been previously treated successfully with anti-estrogen therapy and progressed after achieving initial clinical benefit before progressing on at least 2 such regimens. We hypothesize that these long-term estrogen deprived tumors will be hypersensitive to the effects of Estrace and this will translate into clinical response, resulting in the ability to respond to further endocrine treatment, an aromatase inhibitor, in this heavily endocrine pre-treated population. Our overall goal will be to evaluate the response rate to Estrace as well as the expected progression free survival with further endocrine treatment (an aromatase inhibitor) with the future plan of de-escalating the Estrace dose, thereby minimizing toxicities in this particularly targeted population.

During the first year of funding, we have focused on building the clinical infrastructure for the conduct of this multi-institutional clinical trial with Fox Chase Cancer Center (FCCC) serving as the functional "central operations center" for the adverse event monitoring, regulatory surveillance and control, and quality assurance. In collaboration with FCCC Biostatistics department, we have developed an electronic database for the clinical information acquisition including patient enrollment logs, demographics, health history, physical exams, prior treatment(s), concomitant medications, drug compliance, adverse events/toxicities, clinical responses, clinical labs and quality of life assessments. We have also sought and successfully secured funding for this investigator-initiated clinical trial as a non-restricted grant from Astra-Zeneca Pharmaceuticals to support the clinical trial operations. Together with Johns Hopkins University Kimmel Cancer Center, we anticipate enrollment beginning early 2008.

**Proteomic analysis of phosphotyrosine-containing protein complexes during estrogen-induced proliferation and apoptosis in MCF-7 human breast cancer cells**

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We have used a panel of MCF-7 cells *in vitro* that have been E2-deprived (ED) for several years to replicate resistance to aromatase inhibitors. The parental MCF-7 cell line responds to estradiol (E2) with growth, whereas E2 causes rapid apoptosis in estrogen-deprived clonal variant MCF-7:5C cells. This study aimed to identify early signaling pathways underlying the low-dose estrogen-induced apoptosis in the MCF-7:5C breast cancer cell line, which may have significant implications in designing novel therapies for anti-estrogen drug resistant breast cancers. To identify these pathways we have established a process to use immunoprecipitation to isolate protein signaling complexes involved in estrogen or growth factor signaling, thereby fractionating the cells' proteome. An antibody recognizing tyrosine phosphorylated proteins was used to pull-down unique complexes of proteins in estrogen-treated vs. -untreated cells, followed by 1D-SDS PAGE. Bands determined to be unique for each treatment condition, in duplicate, after 2 hours treatment with E2, were excised and subjected to mass spectroscopic analysis (MS/MS). The results were analyzed using the iProXpress system, facilitating functional annotation of identified proteins, and Ingenuity® Systems pathway analysis software, allowing the proteins to be mapped to known cell signaling pathways. Here, we have identified 25 unique protein targets in E2-treated MCF-7 cells, and 15 unique protein targets in E2-treated MCF-7:5C cells. In E2-treated MCF-7 cells, 7 proteins are associated with a signaling network with nodes centered on H-RAS and coatamer A/B, and 17 proteins are associated with a signaling network with nodes centered around TNF-α and IL-1β, both networks of which are involved strongly in cancer. In contrast, analysis of E2-treated MCF-7:5C cells revealed that 9 proteins are associated with a signaling network with nodes centered around retinoic acid, NF-κB, PTEN, and p38 MAPK, and 3 proteins are associated a signaling network with nodes centered around hsp70, hsp90, and cMyc; the former network has been found in cancer signaling, and tissue development signaling, where as the latter network has been associated with endocrine system development/function and lipid metabolism. The next steps in this study are to confirm the existence of these protein complexes by immunoprecipitation and western blot analysis with specific antibodies, and to determine if siRNA targeting of relevant activating pathways affects the E2-induced apoptotic response.